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14. ABSTRACT: The purpose of the project is to characterize the breast cancer in women affected with Neurofibromatosis type 1 (NF1) in a multi-institutional setting. **Aim 1** assessed the incidence of breast cancer in this cohort and the clinical features of NF1 associated with breast cancer and other cancers. A total of 423 cases of NF1 women have been reviewed. History of breast cancer was found in 20. Family history of cancer or breast cancer is associated with personal breast cancer. Neither cutaneous neurofibroma burden nor family history of NF1 was found to be associated with the personal breast cancer. Malignant peripheral nerve sheath tumor (MPNST) is associated with plexiform neurofibromas. Learning disability is associated with CNS tumor and/or optic glioma (OPG). European Americans (EA) are more likely to develop CNS tumor and/or OPG than African Americans (AA). **Aim 2** investigated the NF1 gene germline mutations in women with breast cancer. Germline NF1 mutation has been investigated for 14 women. The types of mutations were not significantly different from the general NF1 population. Germline exome sequencing (WES) has been completed for 14 NF1 women with history of breast cancer and 42 female control subjects. WES has not identified any deleterious mutation in high penetrance breast cancer genes. **Aim 3** IHC analysis has been completed for the selected signaling pathway proteins and growth factor receptors. Nine out of 14 breast cancer specimens from women with NF1 are found to be HER2/neu positive. This rate is significantly higher than the sporadic breast cancers. Ten of these specimens have yielded enough DNA to undergo genome-wide copy number (CN) and LOH analysis. A significant number of the sample has *ERBB2* CN gain in comparison to sporadic cancers. LOH on 16p11.2 encompassing 2.66 Mb is observed in 7 samples. LOH on Xq11.1encompassing 1.95 Mb is observed in 7 samples. **Aim 4**. NF1 inactivation results in human mammary epithelial cells (HMEC) senescence; p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC; p53 inactivation provides an initial growth advantage to HMEC with a consequent large number cell death; Overexpression of K-Ras V12 does not transform p53 inactivated HMEC.

15. SUBJECT TERMS

NF1; breast cancer; signaling pathways; germline mutation; somatic mutation; FFPE; IHC; WES; LOH; copy number variation; NF1 knockdown cells; senescence, Ras

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1. INTRODUCTION

The occurrence of breast cancer is increased in women affected with Neurofibromatosis type 1 (NF1). This study is aimed at identifying an accurate incidence of breast cancer in this group of women in a multi-center collaborative environment. There are 4 specific aims. **Aim 1** is to confirm the increased breast cancer risk in women with NF1. All the participating centers, Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), Children's National Medical Center in D.C. (CNMC), and Johns Hopkins University (JHU), have reviewed the medical records of women affected with NF1. Clinical data were analyzed to identify clinical features associated with the occurrence of breast cancer. Clinical features were also analyzed for their association with other type of cancers in this study. At the same time, women with a history of breast cancer were recruited to donate blood and their archived tumor (FFPE) specimen. **Aim 2** is to analyze the germline *NF1* gene and the whole exome in the subjects with a history of breast cancer. The *NF1* mutations identified were analyzed for genotype-breast cancer correlation. WES was attempted to identify breast cancer predisposition in addition to the *NF1* gene mutation. **Aim 3** is to determine if NF1 associated breast cancers have unique signaling pathways or molecular signatures. Immunohistochemistry (IHC) study of the signaling pathways was performed on archived tumor blocks. Genome-wide copy number and loss of heterozygosity (LOH) analysis were performed on these tumor specimens. **Aim 4** is to study the phenotype of *NF1* knockdown in primary mammary epithelial cells, specifically focused on the senescence effect due to Ras activation. This study attempted to provide information in determining when and how to screen for breast cancer in this group of women. It has also shed light on the molecular mechanisms of breast cancer in *NF1* deficient human subjects.

One major change in this project is that in December, 2014, the principle investigator who designed and initiated this project, Dr. Xia Wang, left HFHS in Detroit and moved to Moffitt Cancer Center in Tampa, Florida. The PI is transferred to Dr. Dhananja Chitale, one of the major collaborators in HFHS. With the consent from HFHS and Dr. Chitale, Dr. Wang continued to manage this project till the end.

2. **KEY WORDS**

Neurofibromatosis type 1; breast cancer; clinical features; family history; signaling pathway; germline mutation; somatic mutation; formalin fixed and paraffin embedded (FFPE); immunohistochemistry (IHC); whole exome sequencing (WES); loss of heterozygosity (LOH); copy number variation (CNV); *NF1* knockdown cells; senescence, Ras

3. ACCOMPLISHMENTS

Aim 1: To confirm the increased breast cancer risk in women with NF1. To identify any clinical features associated with the risk for breast cancer.

***Task 2*:** Clinical data collection, analysis, patient contact and specimen retrieval

2a. Chart review and data recording in each clinical study site -- 423 cases collected, 20 cases had a personal history of breast cancer. -- Completed (Dec 2013)

2b. Aim 1 data analysis in HFHS -- Completed (Sept 2015)

2c. Obtain consent, archived tumor specimens and blood, obtain previous genetic testing results. --Completed (May 2014)

2d. Recruit more breast cancer cases outside NF clinics in each study site, obtain records, archived tumor specimens and blood. -- Completed (May 2014)

2e. Manuscript development for Aim 1 -- Completed (Oct 2015)

Henry Ford Health IT helped to build the secured electronic database for all the participating clinical sites to enter the clinical data onsite. The database has been promptly removed in April 2014 after the final collection of data. The last case was entered by JHU site into the electronic database in February 2014.

Comprehensive analysis has been completed for the family history, clinical features and cancers in 423 cases of women affected with NF1. Personal history of breast cancer was identified in 20 women. This number is lower than expected 50 cases. The number of 50 cases in 500 NF1 women was based on the incidence of 10% in the small cohort of HFHS reported previously.

Manuscript has been accepted by the Journal of Genetic Syndromes and Gene Therapy in March 2016. It is in the section of "Products"

Poster presentation related to Aim 1:

- 6-2013 CTF (Children's Tumor Foundation) annual conference "The Incidence of Cancer in Women with Neurofibromatosis Type 1" Renée Tousignant, MS, MSC, Xia Wang, MD, PhD, FACMG & Albert Levin, PhD
- 6-2014 CTF annual conference "The Incidence of Neoplasms in 424 Women with Neurofibromatosis Type 1" Xia Wang, MD, PhD, Renee Tousignant, MS, CGC *Henry Ford Health Group*; Albert Levin, PhD, Henry *Ford Health System*; Bruce Korf, MD, PhD, *University of Alabama at Birmingham*; Jaishri Blakeley, MD, *Johns Hopkins University*; Maria Acosta, MD, *Children's National Medical Center*

Aim 2: To analyze germline *NF1* gene of the subjects with history of breast cancer. The mutations identified will be analyzed for genotype-phenotype correlation; Germline whole exome sequencing (WES) will be carried out on DNA from lymphocytes.

***Task 3*:** *NF1* gene mutation testing and mutation data analysis

3a. Consent subjects and send blood for clinical germline *NF1* gene analysis. -- Completed (May 2014)

3b. *NF1* genotype data analysis (14 cases were collected and analyzed) -- Completed (June 2014)

3c. Consent and germline whole exome sequencing (WES) – 14 cases (This plan was added in July, 2013) -- Completed (May 2014)

WES on 42 control germline lymphocytes DNA samples (This plan was added in Feb 2015) -- completed (July 2015)

3d. *Collaboration with Dr. Gareth Evens from U.K. – This plan has been cancelled -- cancelled in 2014*

3e. WES data annotation and analysis (preliminary) – Completed (March 2015)

WES data final analysis on 14 cases and 42 controls -- Completed (Jan 2016)

3f. Manuscript development for Aim 2 -- 50% completed

3g. *Sanger sequencing confirmation for clinical actionable mutations identified by WES – It is canceled since no clinical actionable mutation is identified.*

***NF1* gene (germline) analysis:**

A total of 16 NF1 women were recruited to donate blood and tumor specimen for analysis. HFHS site has recruited 9 women via self-referral, referral from NF patient advocate groups, such as Children's Tumor Foundation (CTF) and NF Michigan Chapter or health care providers outside HFHS. These women underwent consent via telephone discussion. Their health care providers coordinated the specimen collection and shipment. The relevant medical records were collected. HFHS site has also recruited additional 3 women from genetics or neurofibromatosis outpatient clinic in HFHS. CNMC site recruited one woman and had her consented to donate blood. JHU site recruited 3 women and had them consented to donate blood. A tumor specimen from one of the women from JHU site was available to be retrieved. No woman was recruited from UAB site.

The blood and breast tumor specimens (when available) were collected along with medical history, family history and pathological reports. The information collected includes age, ethnicity, age at menarche, number of café au lait macules, skin fold freckling, Lisch nodules on the irises, number of dermal neurofibromas, number of plexiform neurofibromas, history of optic gliomas, malignant peripheral nerve sheath tumor, bony dysplasia, macrocephaly, short stature and learning disability. Additional information regarding neoplasia collected includes occurrence of any malignant solid tumor, malignant hematological disorder, malignant or benign tumor of the central nervous system (CNS). For breast cancer, the pathological type, stage and age at diagnosis are collected when available. Family history information includes NF1, malignant neoplasm, CNS tumor, and

number of relatives with breast cancer. Genetic test results on *NF1* gene and other high penetrance breast cancer gene, e.g. *BRCA1* and *BRCA2* were collected when available.

Blood specimens were sent from each clinical site to the Medical Genomic Laboratory at the University of Alabama at Birmingham (UAB) for comprehensive *NF1* gene analysis, as previously described (Messiaen et al, 2000, PMID: 10862084). *NF1* mutations are described following recommendations of the Human Genome Variation Society using NM_000267.3 as the reference sequence. Exon numbering uses the historical numbering used by the NF1 consortium, followed by the NCBI numbering in square brackets.(Table 1)

NF1 mutation has been discovered in all cases except one women from HFHS site. This case without a *NF1* mutation was excluded from further analysis since we were unable to confirm her family history of NF1 in details. Her family history served as one of the two critical clinical diagnostic criteria for her case. Without the family history, this woman would not fit the diagnostic criteria for NF1. In addition, based on the limited information from *NF1* gene analysis, the women recruited from CNMC is likely to be the same person recruited from JHU. Therefore only one of the two samples was used for further analysis. The last case of *NF1* gene analysis was completed in June 2014. Because the tests were performed by UAB, a CLIA credentialed lab, the results have been given to the women to keep or share with their health care providers.

In 2013, Sabbagh et al. published genotypes of 565 unselected NF1 patients. In this French cohort, 65% of the patients show a truncating mutation, 6.5% an in-frame splicing mutation, and 7.5% a missense mutation. Our cohort of 13 breast cancer NF1 patients shows that 11/13 carrying a truncating mutation (5 carry an out of frame splice mutation; 6 have a truncating mutation due to a frameshift, nonsense or out-of frame copy number change), 1/13 have an in frame splice mutation and 1/13 have a missense mutation. Therefore, the mutational spectrum in our NF1 patients with breast cancer did not differ from the unselected large cohort described by Sabbagh (Chi square, 2- tailed: p=0.23, p=0.44, resp. p=0.59).

The histological types and subtypes of breast carcinoma were available in 11 cases in our cohort. Ten cases are estrogen receptor positive, likely luminal A or B type based on the receptor status. Only one case is ER negative with HER2 over-expression. Basal-like type (usually manifested as ER/PR/HER2 negative) was not found. In the general population, ER-negative tumors represent 20-30% of all breast cancers, with a higher proportion in younger women (Chu et al., 2002). Triple negative tumors account for 15% - 25% of all breast cancers (Cleator et al., 2007; Yanagawa et al., 2012). Amplification or over-expression of the *ERBB2* gene occurs in approximately 15-30% of breast cancers (Mitri et al., 2012; Burstein 2005). Surveillance, Epidemiology, and End Results (SEER) Registry shows that lobular carcinoma represent 9-15% of breast cancer in the United States (Li et al., 2003). In women with NF1, invasive lobular type was found in 3 of 14 cases of breast cancer by Sharif et al. (2007), 1 of 10 cases by Wang et al. (2012), and 0 of 4 cases by Madanikia et al. (2012), resulting in a ratio of 14.3 %.

Over all, based on our limited number of cases in this section, breast cancer in women with NF1 does not show a propensity for a certain type, except basal-like subtype, appears to be under-represented, although receptor status is missing in a significant number of the tumors in this cohort.

The clinical features and NF1 mutation is illustrate as **Table 1**.

We have yet to complete the manuscript including this section of the work.

We have recruited 14 cases of NF1 women with history of breast cancer, lower than the goal of 50 cases originally planned. There is a significant low rate of breast cancer in UAB site. In addition, recruitment rate is low for the patients from the neurofibromatosis clinics as a result of lost follow up and death. There was not enough manpower in CNMC, JHU, and UAB to recruit participants from sources outside clinics.

Table 1. *NF1* gene mutation, breast cancer pathology and cancer history of the 14 NF1 patients studied

ID	Breast Cancer								Personal History Other Cancer	Family History Cancer	Family History NF1	Female Relative breast cancer and NF1	Mutation (DNA level; RNA level; protein level)		
	Age Diag-nosis (years)	Age Men-arche (years)	Pathology										Exon	Type	Description
			Type	ER	PR	Her2	Ki-67	Proposed subtype							
1	56	13	IDC	NA	NA	NA	NA	NA	--	--	De novo	--	33 [42]	OOF skipping exon 33 [42] - PSC	c.6364G>A; r.6085_6364del; p.Val2029Lysfs*7
2	53	NA	IDC	+	+	--	NA	Luminal A/B	Carcinoid tumor; Pheochromo-cytoma	Breast; Ovary; Esophagus	Inherited	--	10c [14]	Frame-shift - PSC	c.1541_1542delAG; r.1541_1542delag; Gln514Argfs*43
3	60	16	DCIS	+	+	NA	NA	Luminal B	--	Breast; Ovary; Pancreas; gastrium	De novo	--	37 [46]	IF skipping exon 37 [46]	c.6792C>G; r.6757_6858del; p.Ala2253_Lys2286del
4	49	NA	IDC	--	--	+	NA	HER2	Cervical cancer	Breast; Pheochromo-cytoma	Inherited	NA	15 [20]	Non-sense - PSC	c.2398G>T, r.2398g>u; p.Glu800*
5	45	14	NA	NA	NA	NA	NA	NA	--	--	De novo	--	Intron 26 [34]	OOF splicing - PSC	c.4515-20_4515-18delAAG; r.4514_4515ins4515-14_4515-1; p.Arg1505Serfs*53
6	47	11	IDC	+	+	--	NA	Luminal A/B	--	Breast; Prostate	NA	--	16 [21]	Trunca-tion and low level OOF splicing - PSC	c.2621_2634dupAGGGTTCTATGATT; r.2621_2634dupaggguucuaugauu and r.2618_2850del; p.Ser879Argfs*4 and p.Lys874Phefs*4
7	39	NA	IDC	+	+	NA	NA	Luminal A/B	--	--	Inherited	--	29-30 [38-39]	copy number variant - PSC	c.(5045_5337)_(5625_5796)del; r.5206_5749del; p.Gly1737Leufs*3
8	41	11	IDC	+	--	Eq	NA	Luminal A/B	--	Lung; Colon; Pheochromo-cytoma	Inherited	--	12a [16]	Mis-sense	c.1733T>G; r.1733u>g; p.Leu578Arg
9	49	14	IDC	+	--	+	3 (20%)	Luminal B	--	Breast	NA	NA	Intron 31 [40])	OOF splicing - PSC	c.5943+1G>T; r.5901_5943del; p.Met1967Ilefs*10
10	44	NA	IDC	+	+	--	3 (15%)	Luminal B	--	Breast; Colon; Lung; Prostate	Inherited	NA	16 [21]	Frame-shift - PSC	c.2728_2729delGG; r.2728_2729delgg; p.Gly910Thrfs*8
11	58	NA	NA	NA	NA	NA	NA	NA	--	--	NA	NA	28 [37]	Frame-shift -	c.4910_4911delTT; r.4910_4911delyy; p.Phe1637Serfs*3

Table 1. *NF1* gene mutation, breast cancer pathology and cancer history of the 14 NF1 patients studied

ID	Breast Cancer								Personal History Other Cancer	Family History Cancer	Family History NF1	Female Relative breast cancer and NF1	Mutation (DNA level; RNA level; protein level)		
	Age Diag-nosis (years)	Age Men-arche (years)	Pathology										Exon	Type	Description
			Type	ER	PR	Her2	Ki-67	Proposed subtype							
														PSC	
12	52	13	IDC	--	--	+	NA	HER2	--	Breast; Gastrium	De novo	--	30 [39]	Frame-shift - PSC	c.5667dupT; r.5667dupu; p.Ile1890Tyrfs*2
13	47	13	IDC	24%	2%	--	59%	Luminal B	--	Breast; Ovary	Inherited	+	9 [11]	deep intronic splice mutation - PSC	c.1260+1604A>G,r.1260_1261ins1260+1605_1260+1646; p.Ser421_Val2818delinsLeuThrThr*
14	42	13	IDC	+	+	--	NA	Luminal A/B	--	Breast; Ovary	Inherited	+	9 [11]	deep intronic splice mutation - PSC	c.1260+1604A>G, r.1260_1261ins1260+1605_1260+1646; p.Ser421_Val2818delinsLeuThrThr*

IDC: Invasive ductal carcinoma
DCIS: Ductal carcinoma in situ
NA: Information not available
ER: Estrogen receptor status
PR: Progesterone receptor status
HER2: Human epidermal growth factor receptor 2 expression
Ki-67: Ki-67 proliferation marker
Eq: Equivocal
OF: Out-of-frame
IF: In-frame
PSC: premature stopcodon

WES (germline) analysis:

By providing the updated WES specific information, participants who have already undergone germline *NF1* gene testing were re-consented for the additional germline WES analysis. Participants recruited after the decision to add WES analysis (July 2013) were consented by providing WES related information and *NF1* gene testing information.

Germline lymphocytes DNA from 14 cases (NF1 women who have had a diagnosis of breast cancer) has completed **WES (Whole exome sequencing analysis)** by AGTC, the genomic core lab in Wayne State University (WSU) in October, 2014. The WES employed Illumina HiSeq 2500, Nextera Rapid Capture Exome protocol and 2x 100 bp paired end rapid run.

Preliminary analysis on WES data was processed by Bioinformatics in WSU in October, 2014. It utilized Illumina CASAVA software, FastQC, alignment to human reference genome hg19, SNP calling and filtering using Genome Analysis Toolkit. QC analysis on some of the samples was suboptimal. Targeted exploration did not reveal mutations in high risk hereditary breast cancer genes, including *BRCA1* or *BRCA2* gene. We were unable to generate any significant results.

Preliminary analysis on WES data was attempted again by Brandon Shaw, Ph.D. and Xia Wang, Ph.D. from December 2014 to March 2015. We have utilized the Omicia, Opal Research™ clinical interpretation program for NGS data. It consists of the following: Sequencing Quality Assessment, Automated Genome Annotation (drawing annotations from data sources including OMIM, ClinVar, and COSMIC), Predicted Pathogenicity Scoring (including SIFT, PolyPhen, CADD, MutationTaster, PhyloP and the Omicia Variant Score). The Omicia Score is a meta-classifier that combines scores from the mentioned variant scoring algorithms. The Omicia score ranges from 0 to 1. Less than 0.5 would indicate that a variant is likely benign. Greater than 0.5 suggest that a variant is likely to be damaging or deleterious, with higher confidence at values closer to 1. This analysis did not reveal any mutations in high risk hereditary breast cancer genes, including *BRCA1* or *BRCA2* gene. This analysis did not reveal any genes or mutations that were shared by those NF1 women with breast cancer history. In addition, the sequencing data appeared to have significant artifacts.

WES control samples: It was then determined that **sequencing controls subjects** with the same platform may alleviate the false positives on variant calling. As controls, germline lymphocytes DNA were randomly selected from 42 de-identified samples in Henry Ford Hospital molecular diagnostic laboratory. These DNA samples were from women who were presumably healthy and undergoing prenatal hereditary genetic screening tests. These samples underwent WES in Wayne State University (WSU) AGTC genomic core lab in June, 2015. WSU lab reported using the same platform and capture kits, Illumina HiSeq 2500, Nextera Rapid Capture Exome protocol and 2x100 bp paired end rapid run. Initial QC for 21 samples was not satisfactory so that they were re-sequenced again which resulted in satisfactory QC. The other 21 samples also passed the QC tests.

Final WES comprehensive analysis was completed by Moffitt Cancer Center (MCC) Bioinformatics Core in January 2016 using the data of the 14 NF1 women (cases) and 42 controls. These include QC report and sequencing data analysis. The analysis sugested that WES of the cases and the controls appeared to have used different capture kits and different sequencing platforms. However, this finding is inconsistent with the reports from WSU.

Sequence reads were aligned to the reference human genome with the Burrows-Wheeler Aligner (BWA). Duplicate identification, insertion/deletion realignment, quality score recalibration, and variant identification was performed with PICARD (http://picard.sourceforge.net/) and the Genome Analysis ToolKit (GATK). Genotypes (reference and variant) at variant positions were determined using GATK on all samples simultaneously. Sequence variants were annotated to determine genic context (ie, non-synonymous, missense, splicing) using ANNOVAR and summarized using spreadsheets and a genomic data visualization tool,

VarSifter. Additional contextual information was incorporated, including allele frequency in other studies such as 1000 Genomes and the NHLBI Exome Sequence Project, *in silico* function impact predictions, as well as observed impacts from databases such as ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), and The Cancer Genome Atlas (TCGA). Sample stratification was assessed using multidimensional scaling via R and Plink. The samples were separated using genotypes from variants seen at minor allele frequency > 20%. The 1000 Genomes Phase 1 version 3 dataset was used as reference. Somatic mutations were enriched by only considering coding variants observed at 1% or less in 1000 Genomes and 5% or less in an internal cohort of normal samples. To ensure high quality, variants were only considered with GQ score >=15 and VQSR Tranche level <= 99.0. Differences in mutation rates were assessed at the position and gene (truncating mutations) levels using the Fisher Exact test. Multiple testing was corrected using the Benjamini-Hochberg method.

WES data analysis generated a significant number of false positive artifacts. After applying increased stringency, a significant number of false positive artifacts have been excluded. Since the majority of the cases are reported to be Caucasians as ancestry, the analysis was focused on the individuals of European ancestry. The samples of African or mixed ancestry were excluded to avoid population specific false positive variants. Out of 56 samples (14 cases and 42 controls), ancestry cluster analysis showed 21 were non-European, including 3 NF1 cases. This resulted in 11 NF1 cases and 24 controls. Non-European samples were not analyzed because of the small number which makes statistical analysis not possible. Variants were categorized into the following categories: nonsynonymous single nucleotide change (nsy-SNV), non-frameshift deletion or insertion (nfs-InDel), splicing variant (SpV), and any variant resulting truncation, i.e. nonsense single nucleotide change (ns-SNV) or frameshift deletion or insertion (fs-InDel), stop codon gain (SCG) and stop codon loss (SCL). The variant analysis was reported in three categories: 1. Variants at the position level; 2. All variants collapsed to the gene level; 3. Truncating variants.

1. A total of 58 variants were found to be more prevalent in the test group by Fisher's test, p=<0.05
2. Variants in 84 genes are found to be more prevalent in the case group, p=<0.05.
3. In the truncation mutation category, 3 mutations in 3 separate genes reached significance, p=<0.05.

However, none of these genes or variants has reached significance after multi-test analysis.
(**Table 2, 3, 4**).

Table 2. Variants based on positions													
Chr	Posi-tion	Gene	Annotation	NF1				Control				Fisher's Tests	
				Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
1	244583585	ADSS	nonsynonymous_SNV:ADSS:p.K226R	7	4	0	0	24	0	0	0	0.0063025 2	1
8	143816828	C8orf55	nonsynonymous_SNV:C8orf55:p.G200R	6	5	0	0	23	1	0	0	0.0071157 5	1
X	72783170	CHIC1	nonframeshift_deletion:CHIC1:p.17_18del	6	5	0	0	20	1	0	3	0.0112161 7	1
6	16327915	ATXN1	nonframeshift_insertion:nonframeshift_insertion:ATXN1:ATXN1:p.Q208delinsQQQ:p.Q208delinsQQQ	0	7	3	1	6	3	12	3	0.0113636 4	1
1	145075775	PDE4DIP	nonsynonymous_SNV:PDE4DIP:p.P30S	5	5	0	1	22	2	0	0	0.0138879 8	1
21	10942756	TPTE	stopgain_SNV:stopgain_SNV:stopgain_SNV:TPTE:TPTE:TPTE:p.R211X:p.R191X:p.R229X	6	5	0	0	3	21	0	0	0.0145902 9	1
21	10942924	TPTE	nonframeshift_deletion:nonframeshift_deletion:nonframeshift_deletion:TPTE:TPTE:TPTE:p.202_203del:p.182_183del:p.220_221del	6	5	0	0	3	21	0	0	0.0145902 9	1
20	45131155	ZNF334	nonsynonymous_SNV:nonsynonymous_SNV:ZNF334:ZNF334:p.R275C:p.R237C	6	3	0	2	24	0	0	0	0.0153958 9	1
15	71276480	LRRC49	nonframeshift_deletion:nonframeshift_deletion:nonframeshift_deletion:LRRC49:LRRC49:LRRC49:p.352_352del:p.308_308del:p.357_357del	0	11	0	0	9	14	0	1	0.0172451 5	1
7	128505233	ATP6V1F	nonsynonymous_SNV:ATP6V1F:p.P73L	6	4	0	1	23	1	0	0	0.0190184 6	1
1	155261649	PKLR	nonsynonymous_SNV:nonsynonymous_SNV:PKLR:PKLR:p.V506I:p.V475I	7	3	0	1	24	0	0	0	0.0200534 8	1

Table 2. Variants based on positions													
Chr	Position	Gene	Annotation	NF1				Control				Fisher's Tests	
				Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
3	190573136	GMNC	nonsynonymous_SNV:GMNC:p.R318H	7	3	0	1	24	0	0	0	0.02005348	1
13	47345630	ESD	nonsynonymous_SNV:splicing:ESD:ESD:p.G257D:splicing	7	3	0	1	24	0	0	0	0.02005348	1
19	56934722	ZNF583	nonsynonymous_SNV:nonsynonymous_SNV:nonsynonymous_SNV:ZNF583:ZNF583:ZNF583:p.R232K:p.R232K:p.R232K	7	3	0	1	24	0	0	0	0.02005348	1
3	130447529	PIK3R4	splicing:PIK3R4:c.1586-1G>T	1	2	0	8	13	0	0	11	0.025	1
1	16349137	CLCNKA	nonsynonymous_SNV:nonsynonymous_SNV:CLCNKA:CLCNKA:p.R8H:p.R8H	8	3	0	0	24	0	0	0	0.02521008	1
1	180886140	KIAA1614	nonsynonymous_SNV:KIAA1614:p.R301C	8	3	0	0	24	0	0	0	0.02521008	1
2	178494173	PDE11A	nonframeshift_insertion:PDE11A:p.P478delinsSP:p.P672delinsSP:p.P922delinsSP:p.P564delinsSP	3	8	0	0	0	24	0	0	0.02521008	1
3	130104088	COL6A5	nonsynonymous_SNV:COL6A5:p.A581V	8	3	0	0	24	0	0	0	0.02521008	1
3	135720663	PPP2R3A	nonsynonymous_SNV:PPP2R3A:p.N108S	8	3	0	0	24	0	0	0	0.02521008	1
6	35259397	ZNF76	nonsynonymous_SNV:ZNF76:p.R272C	8	3	0	0	24	0	0	0	0.02521008	1
6	155153307	SCAF8	nonsynonymous_SNV:SCAF8:p.S865N	8	3	0	0	24	0	0	0	0.02521008	1
6	161127501	PLG	nonsynonymous_SNV:nonsynonymous_SNV:PLG:PLG:p.K38E:p.K38E	8	3	0	0	24	0	0	0	0.02521008	1
7	99521208	GJC3	nonsynonymous_SNV:GJC3:p.A267V	8	3	0	0	24	0	0	0	0.02521008	1

Table 2. Variants based on positions													
Chr	Posi-tion	Gene	Annotation	NF1				Control				Fisher's Tests	
				Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
7	100218631	TFR2	nonsynonymous_SNV:nonsynonymous_SNV:TFR2:TFR2:p.R752H:p.R581H	8	3	0	0	24	0	0	0	0.02521008	1
7	135418881	FAM180A	nonsynonymous_SNV:FAM180A:p.D122N	8	3	0	0	24	0	0	0	0.02521008	1
9	35295880	UNC13B	nonsynonymous_SNV:UNC13B:p.D238E	8	3	0	0	24	0	0	0	0.02521008	1
9	79938036	VPS13A	nonsynonymous_SNV:VPS13A:p.R1923C:p.R1962C:p.R1962C:p.R1962C	8	3	0	0	24	0	0	0	0.02521008	1
10	96484145	CYP2C18	nonsynonymous_SNV:CYP2C18:CYP2C18:p.R335Q:p.R276Q	8	3	0	0	24	0	0	0	0.02521008	1
11	117063027	SIDT2	nonsynonymous_SNV:SIDT2:p.A644S	8	3	0	0	24	0	0	0	0.02521008	1
15	38228620	TMCO5A	nonsynonymous_SNV:TMCO5A:p.Q32H	8	3	0	0	24	0	0	0	0.02521008	1
15	90213343	PLIN1	nonsynonymous_SNV:nonsynonymous_SNV:PLIN1:PLIN1:p.V156L:p.V156L	8	3	0	0	24	0	0	0	0.02521008	1
17	33430313	RAD51D	nonsynonymous_SNV:RAD51D:RAD51D:RAD51D:p.E233G:p.E253G:p.E121G	8	3	0	0	24	0	0	0	0.02521008	1
19	1924189	SCAMP4	nonsynonymous_SNV:SCAMP4:p.P199L	8	3	0	0	24	0	0	0	0.02521008	1
19	48876829	SYNGR4	nonsynonymous_SNV:SYNGR4:p.M50T	8	3	0	0	24	0	0	0	0.02521008	1
19	57868483	ZNF304	nonsynonymous_SNV:ZNF304:p.A416T	8	3	0	0	24	0	0	0	0.02521008	1
22	38627339	TMEM184B	splicing:TMEM184B:splicing	8	3	0	0	24	0	0	0	0.02521008	1

Table 2. Variants based on positions													
Chr	Posi-tion	Gene	Annotation	NF1				Control				Fisher's Tests	
				Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
4	2172456	POLN	nonsynonymous_SNV:POLN:p.S502G	7	4	0	0	23	1	0	0	0.02582001	1
10	134999646	KNDC1	nonsynonymous_SNV:KNDC1:p.T265I	1	3	0	7	11	1	0	12	0.0269230 8	1
8	146062872	ZNF7	nonsynonymous_SNV:ZNF7:p.A76V	8	3	0	0	23	0	0	1	0.0275735 3	1
17	66878099	ABCA8	nonsynonymous_SNV:ABCA8:p.C1244Y	8	3	0	0	23	0	0	1	0.0275735 3	1
19	55086249	LILRA2	nonsynonymous_SNV:nonsynonymous_SNV:LILRA2:LILRA2:p.L135S:p.L135S	8	3	0	0	22	0	0	2	0.0302419 4	1
11	6341397	PRKCDBP	nonsynonymous_SNV:PRKCDBP:p.A104T	1	2	0	8	11	0	0	13	0.0329670 3	1
17	21318821	KCNJ12,KCNJ18	nonsynonymous_SNV:nonsynonymous_SNV:KCNJ12:KCNJ18:p.E56A:p.E56A	0	11	0	0	9	15	0	0	0.0331082 2	1
14	104644142	KIF26A	nonsynonymous_SNV:KIF26A:p.A1673T	3	2	0	6	20	0	0	4	0.0333333 3	1
19	55858576	SUV420H2	nonsynonymous_SNV:SUV420H2:p.R383H	3	2	0	6	20	0	0	4	0.0333333 3	1
6	69949092	BAI3	nonsynonymous_SNV:BAI3:p.V930I	4	2	0	5	24	0	0	0	0.0344827 6	1
19	55086029	LILRA2	nonsynonymous_SNV:nonsynonymous_SNV:LILRA2:LILRA2:p.P111R:p.P111R	8	3	0	0	20	0	0	4	0.0367074 5	1
22	38483155	BAIAP2L2	nonframeshift_insertion:BAIAP2L2:p.N412delinsTPMN	1	9	0	1	9	7	0	8	0.0367429 9	1
5	65474560	SREK1	nonsynonymous_SNV:nonsynonymous_SNV:SREK1:SREK1:p.N464S:p.N580S	4	2	0	5	23	0	0	1	0.0369458 1	1
19	13875821	MRI1	nonsynonymous_SNV:nonsynonymous_SNV:MRI1:MRI1:p.R90L:p.R90L	0	1	0	10	23	0	0	1	0.0416666 7	1

Table 2. Variants based on positions													
Chr	Posi-tion	Gene	Annotation	NF1				Control				Fisher's Tests	
				Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
6	31106500	PSORS1C1	frameshift_insertion:PSORS1C1:p.P38fs	2	7	2	0	13	6	5	0	0.04182195	1
1	1961584	GABRD	nonsynonymous_SNV:GABRD:p.R408C	3	3	0	5	9	0	0	15	0.0439604	1
14	23744800	HOMEZ	nonframeshift_deletion:HOMEZ:p.544_545del	4	5	0	2	2	19	0	3	0.04916004	1
8	144990784	PLEC	nonsynonymous_SNV:PLEC; p.T4406M:p.T4539M:p.T4402M:p.T4370M:p.T4388M:p.T4429M:p.T4380M:p.T4402M	4	2	0	5	19	0	0	5	0.05	1
14	52735290	PTGDR	nonsynonymous_SNV:PTGDR:p.A253G	4	2	0	5	19	0	0	5	0.05	1

Table 3. Variants based on genes.										
	NF1				Control				Fisher's Tests	
Gene	Ref	Var	Other	NA	Ref	Var	Other	NA	fisher_p	fisher_q
CACNA1A	11	0	0	0	6	18	0	0	2.97E-05	0.24175593
NF1	3	8	0	0	23	1	0	0	5.69E-05	0.24175593
LILRA2	5	6	0	0	23	1	0	0	0.00169797	1
TNS3	5	6	0	0	23	1	0	0	0.00169797	1
BPTF	4	7	0	0	21	3	0	0	0.00389363	1
LOXHD1	4	7	0	0	21	3	0	0	0.00389363	1
ACPT	11	0	0	0	12	12	0	0	0.00547884	1
ADSS	7	4	0	0	24	0	0	0	0.00630252	1
ALMS1	4	7	0	0	0	24	0	0	0.00630252	1
GPR112	4	7	0	0	0	24	0	0	0.00630252	1
KIAA0319	7	4	0	0	24	0	0	0	0.00630252	1
NLRP9	7	4	0	0	24	0	0	0	0.00630252	1
C8orf55	6	5	0	0	23	1	0	0	0.00711575	1
BAIAP2L2	2	9	0	0	17	7	0	0	0.00878754	1
CHIC1	6	5	0	0	20	1	0	3	0.01121617	1
ART5	2	9	0	0	16	8	0	0	0.01164257	1
LRRC49	0	11	0	0	10	14	0	0	0.01457706	1
ATP6V1F	6	4	0	1	23	1	0	0	0.01901846	1
CHRNA10	7	3	0	1	24	0	0	0	0.02005348	1
ESD	7	3	0	1	24	0	0	0	0.02005348	1
PKLR	7	3	0	1	24	0	0	0	0.02005348	1
ZNF583	7	3	0	1	24	0	0	0	0.02005348	1
AGRN	6	5	0	0	22	2	0	0	0.02066021	1
AKNAD1	6	5	0	0	22	2	0	0	0.02066021	1
SKIV2L	6	5	0	0	22	2	0	0	0.02066021	1
SYNE1	10	1	0	0	11	13	0	0	0.0233124	1
ABCA8	8	3	0	0	24	0	0	0	0.02521008	1
ABCC1	8	3	0	0	24	0	0	0	0.02521008	1
C1orf38	8	3	0	0	24	0	0	0	0.02521008	1
CDK5RAP1	8	3	0	0	24	0	0	0	0.02521008	1

Table 3. Variants based on genes.										
	NF1				Control				Fisher's Tests	
Gene	Ref	Var	Other	NA	Ref	Var	Other	NA	fisher_p	fisher_q
CHKB	8	3	0	0	24	0	0	0	0.02521008	1
DENND5B	8	3	0	0	24	0	0	0	0.02521008	1
FAM162A	8	3	0	0	24	0	0	0	0.02521008	1
FAM180A	8	3	0	0	24	0	0	0	0.02521008	1
FAM213A	8	3	0	0	24	0	0	0	0.02521008	1
FGD4	8	3	0	0	24	0	0	0	0.02521008	1
GJC3	8	3	0	0	24	0	0	0	0.02521008	1
IVL	8	3	0	0	24	0	0	0	0.02521008	1
LPIN3	8	3	0	0	24	0	0	0	0.02521008	1
NEK2	8	3	0	0	24	0	0	0	0.02521008	1
OR1S2	8	3	0	0	24	0	0	0	0.02521008	1
PIK3CD	8	3	0	0	24	0	0	0	0.02521008	1
POR	8	3	0	0	24	0	0	0	0.02521008	1
PRDX5	8	3	0	0	24	0	0	0	0.02521008	1
QPCTL	8	3	0	0	24	0	0	0	0.02521008	1
RAD51D	8	3	0	0	24	0	0	0	0.02521008	1
RP1	8	3	0	0	24	0	0	0	0.02521008	1
SIDT2	8	3	0	0	24	0	0	0	0.02521008	1
SRBD1	8	3	0	0	24	0	0	0	0.02521008	1
SYNGR4	8	3	0	0	24	0	0	0	0.02521008	1
TFR2	8	3	0	0	24	0	0	0	0.02521008	1
TMCO5A	8	3	0	0	24	0	0	0	0.02521008	1
TMEM184B	8	3	0	0	24	0	0	0	0.02521008	1
TRIM40	8	3	0	0	24	0	0	0	0.02521008	1
UBXN11	8	3	0	0	24	0	0	0	0.02521008	1
UHRF1BP1	8	3	0	0	24	0	0	0	0.02521008	1
WDR41	8	3	0	0	24	0	0	0	0.02521008	1
ZNF334	8	3	0	0	24	0	0	0	0.02521008	1
ZNF76	8	3	0	0	24	0	0	0	0.02521008	1
ADCY10	7	4	0	0	23	1	0	0	0.02582001	1

Table 3. Variants based on genes.										
	NF1				Control				Fisher's Tests	
Gene	Ref	Var	Other	NA	Ref	Var	Other	NA	fisher_p	fisher_q
CHPF	7	4	0	0	23	1	0	0	0.02582001	1
COL18A1	4	7	0	0	1	23	0	0	0.02582001	1
CSF2RB	7	4	0	0	23	1	0	0	0.02582001	1
LYST	7	4	0	0	23	1	0	0	0.02582001	1
METTL19	7	4	0	0	23	1	0	0	0.02582001	1
NRAP	7	4	0	0	23	1	0	0	0.02582001	1
PLA2G3	7	4	0	0	23	1	0	0	0.02582001	1
RGS12	7	4	0	0	23	1	0	0	0.02582001	1
SCAMP4	7	4	0	0	23	1	0	0	0.02582001	1
MIS18BP1	10	1	0	0	12	12	0	0	0.02700059	1
AHNAK2	3	8	0	0	17	7	0	0	0.02705387	1
LUC7L2	8	3	0	0	7	17	0	0	0.02705387	1
ZNF7	8	3	0	0	23	0	0	1	0.02757353	1
PRKCDBP	1	2	0	8	11	0	0	13	0.03296703	1
PLA2R1	11	0	0	0	15	9	0	0	0.03310822	1
SUV420H2	3	2	0	6	20	0	0	4	0.03333333	1
CENPF	11	0	0	0	16	8	0	0	0.03701031	1
FAT2	11	0	0	0	16	8	0	0	0.03701031	1
KIAA0182	11	0	0	0	16	8	0	0	0.03701031	1
NKD2	11	0	0	0	16	8	0	0	0.03701031	1
SCN4A	11	0	0	0	16	8	0	0	0.03701031	1

Table 4. Truncation mutations										
	NF1				Control				Fisher's Test	
Truncated gene	Ref	Var	Other	NA	Ref	Var	Other	NA	p	q
NF1	4	7	0	0	24	0	0	0	4.91E-05	0.03297782
TPTE	6	5	0	0	3	21	0	0	0.01459029	1
COL18A1	5	6	0	0	2	22	0	0	0.02066021	1
PIK3R4	1	2	0	8	13	0	0	11	0.025	1
AIM2	6	4	0	1	19	2	0	3	0.0673683	1
LUC7L2	7	3	0	1	8	16	0	0	0.0679644	1
PSORS1C1	2	9	0	0	13	11	0	0	0.06932306	1
TRIM59	10	0	0	1	14	6	0	4	0.07411898	1
SETBP1	0	10	0	1	7	17	0	0	0.07822417	1

Aim 3: To determine if NF1 associated breast cancers have unique signaling pathways or molecular tumorigenesis characteristics.

***Task 4*:** Tumor specimen molecular analysis by LOH (loss of heterozygosity) and methylation assay for NF1, p53, BRCA1, BRCA2, PTEN, and ATM genes and IHC (Immunohistochemistry) assay for proteins, pMEK, ERK, pERK, AKT, mTOR, p53, PTEN, Her2, Ki67 proteins

4a. Assay validation for LOH/methylation (by MLPA) and IHC:
Due to the limited amount of DNA extracted from the tumor FFPE material, LOH assay is being changed to Affymetrix OncoScan FFPE® Assay which assesses copy number alteration and LOH. Methylation analysis has to be abandoned.

4b. *LOH and copy number changes have been investigated by Affymetrix OncoScan FFPE® Assay which assess copy number alteration and LOH. Methylation analysis has to be abandoned. Please see the section of Task 5.* – Completed (Jan 2016)

IHC for tumor specimens. Number of specimens collected = 16. All protein IHC was completed except Ki-67. No control samples were done. -- Completed (July, 2015)

***4c**. Collaboraton with Dr. Gareth Evens from U.K. was canceled.*

4d. Data analysis and manuscript development for Aim 3. -- 50% completetd

Immunohistochemistry assay has been conducted for p53, mTOR, PTEN, Phospo-MEK 1/2 (Ser221), Phospo-p44/42 MAPK (ERK1/2), P44/42 MAPK (Erk 1/2), AKT (pan), HER2/ERB2. Antibodies were purchased from Cell Signaling Technology.

METHODS:

Antibody selection: All the antibodies were obtained from the Cell Signaling Technology. IHC staining for pMEK (the phosphorylated and activated form of MEK), ERK (the non-phosphorylated ERK) and pERK (the phosphorylated and activated ERK) proteins was performed. To examine PI3K pathway activation, IHC for pAKT (phosphorylated and activated AKT) and mTOR protein was done. Antibodies against p53, PTEN and HER2 proteins were also validated by IHC.

- P53 (75F) Rabbit mAB (Catalog #2527): Nuclear staining
- mTOR (7C10) Rabbit mAB (Catalog #2983): cytoplasmic staining
- Phospo-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP^{TM} Rabbit mAB (Catalog #4370): Nuclear
- Phospo-MEK 1/2 (Ser221) (166F8) Rabbit mAB (Catalog #2338)
- PTEN (D4.3) Rabbit mAB (Catalog #9188)
- AKT (pan) (11E7) Rabbit mAB (Catalog #4685)
- P44/42 MAPK (Erk 1/2) (137F5) Rabbit mAB (Catalog #4695)
- HER2/ERB2(29D8) Rabbit mAB (Catalog #2165)

Ten breast tumor FFPE specimens were available and were collected from the participants who consented to donate their specimens. Germline *NF1* mutation status was known for these 10 women. Additional 6 breast tumor specimens of NF1 women were retrieved from Henry Ford Hospital Biorepository. The clinical diagnosis of NF1 was confirmed for these women based on documented clinical presentations. However, the germline *NF1* mutation status on these women are unknown.

Among these 16 breast tumor FFPE specimens, 14 tumors (originated from 13 women) had enough tissue to undergo IHC analysis.

Dr. Chitale has performed the microdissection to ensure 50% of the specimen contains the tumor tissue. Immunohistochemistry stain was applied using antibodies for p53, mTOR, PTEN, Phospo-MEK 1/2 (Ser221), Phospo-p44/42 MAPK (ERK1/2), P44/42 MAPK (Erk 1/2), AKT (pan), HER2/ERB2. Three tumors were deemed too small to have enough tumor tissue to be assayed. IHC assays were performed to the remaining 14 specimens. DNA extraction was applied to 11 specimens appearing to have enough tissue.

A significantly higher proportion of the specimens (9/14, 64%) showed positive staining for HER2/Neu (Human epidermal growth factor receptor 2), in comparison to the 15-25% in sporadic breast cancer, p=0.02.

A great majority of the tumors were documented in the clinical note as ER positive (8/9, 88%), PR positive (6/9, 66%).

Otherwise, none of other protein staining showed significance.

The findings are illustrated in **Table 5**.

We have collected 16 breast tumor specimens, less than the original goal of 50. The reasons are: 1) Incidence rate of breast cancer in NF1 was lower than originally expected; 2) Tumor sample was not available for a number of women with breast cancer; 3) The size of the tumor was not large enough to have enough tissue to be analyzed; 4) All the sites, except HFHS, did not have enough manpower to engage in collecting more specimens.

IHC was not done on control samples as originally planned. The reason is: 1) The case number was too small and findings were too un-representative to generate any statistical significance, even in the presence of control samples.

Ki-67 IHC was not done due to lack of personnel.

It also has become clear that there will not be enough DNA material for MLPA analysis to assess copy number and LOH. Therefore methylation analysis was canceled. LOH analysis was done by OncoScan FFPE Assay kits. The details are described in the following section of Task 5.

Table 5. Immunohistochemistry analysis for breast cancer in NF1												
Study ID	Surg path #	histology	PHOSPHO-MEK1/2			PHOSPHO-MAPK (Erk 1/2) P44/42			MAPK (Erk 1/2) P44/42			
			Intensity	% positive	H score	Intensity	% positive	H score	Intensity	% positive	H score	
OP-1001	S-7762-06-F	IDC Small tumor	0	0	0	0	0	0	1	90	90	
1070	S01-6732	DCIS only	1	80	80	3	20	60	1	30	30	
OP-1003	S13-8539	IDC	1	100	100	3	20	60	2	65	130	
OP-1004	P12-3709	IDC+DCIS	1	100	100	0	0	0	2	90	180	
OP-1005	W1203-111	DCIS+IDC?	1	100	100	3	2	6	1	100	100	
OP-1006	6198-6B-13	DCIS+IDC	1	50	50	3	5	15	1	95	95	
1299	S03-7282	IDC+DCIS	0	0	0	2	30	60	0	0	0	
OP-1007	S14-5536	small tumor DCIS+IDC	1	100	100	0	0	0	1	100	100	
OP-1008	S-03-21705	DCIS+IDC	1	100	100	3	1	3	1	95	95	
HS06-19153	HS06-19153	IDC	1	50	50	0	0	0	1	70	70	
HS08-3064	HS08-3064	DCIS	1	100	100	3	3	9	2	100	200	
S13-3214-2	S13-3214-3	DCIS	2	100	200	3	70	210	2	100	200	
S13-3214-2	S13-3214-3	IDC	2	100	200	3	40	120	2	100	200	
S14-5942-2	S14-5942-3	IDC	2	100	200	3	30	90	1	40	40	
	S03-7282: ?poor processing leading to false negative results					Sclerosing adensis high p44 expression			S14-5536-1 DCIS 2+ / 95%			
						Infitrating edge has highp44 expression						
	100 OR LESS looks negative											
	Maximum H score: 300											

Table 5. Immunohistochemistry analysis for breast cancer in NF1																		
Study ID	Surg path #	His-tology	PTEN			AKT			mTOR			p53			HER2/NEU		ER	PR
			Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	POS/ NEG		
OP-1001	S-7762-06-F	IDC Small tumor	1	100	100	1	50	50	0	0	0	1	20	20	0	NEG	POS	POS
1070	S01-6732	DCIS only	1	100	100	1	50	50	0	0	0	3	90	270	3	POS	NEG	NEG
OP-1003	S13-8539	IDC	0	0	0	2	100	200	3	40	120	2	80	160	3	POS	POS	POS
OP-1004	P12-3709	IDC+DCIS	1	100	100	1	80	80	1	100	100	1	5	5	1	NEG	POS	POS
OP-1005	W1203-111	DCIS+IDC?	1	100	100	2	100	200	1	100	100	3	95	285	3	POS	POS	NEG
OP-1006	6198-6B-13	DCIS+IDC	1	100	100	1	80	80	1	100	100	3	95	285	3	POS	POS	NEG
1299	S03-7282	IDC+DCIS	0	0	0	0	0	0	0	0	0	0	0	0	0		POS	POS
OP-1007	S14-5536	small tumor DCIS+IDC	1	100	100	2	100	200	0	0	0	0	0	0	1	NEG	POS	POS
OP-1008	S-03-21705	DCIS+IDC	1	100	100	1	100	100	1	90	90	1	5	5	1	NEG	POS	POS
HS06-19153	HS06-19153	IDC			0	1	100	100	1	100	100	2	85	170	3	POS		
HS08-3064	HS08-3064	DCIS	1	100	100	2	95	190	2	100	200	1	30	30	2 TO 3	POS		
S13-3214-2	S13-3214-3	DCIS	1	100	100	1	95	95	2	90	180	3	95	285	3	POS		
S13-3214-2	S13-3214-3	IDC	1	100	100	1	100	100	1	100	100	3	95	285	3	POS		
S14-5942-2	S14-5942-3	IDC	1	100	100	2	100	200	3	90	270	1	2	2	3	POS		
	S03-7282: ?poor processing leading to false negative results		PTEN LOSS in some cases												S13-8539: Heterogenous			
															P12-3709: DCIS sample positive			
	100 OR LESS looks negative														6198-6B-13: ?All DCIS positive			
	Maximum H score: 300																	

Continue …Aim 3: To determine if NF1 associated breast cancers have unique signaling pathway and molecular tumorigenesis characteristics.

Task 5: Next generation cancer gene sequencing on the FFPE tissue including *NF1*, *BRCA1*, *BRCA2*, *TP53*, *PTEN*, and *ATM* genes, and additional breast cancer gene targets including; *CDH1*, *RB1*, *MLL3*, *MAP3K1*, *CDKN1B*, *PIK3CA*, *AKT1*, *GATA3 TBX3*, *RUNX1*, *CBFB*, *AFF2*, *PIK3R1*, *PTPN22*, *PTPRD*, *3F3B1*, *CCND3* and possibly other genes.

5a. *Next generation sequencing on tumor FFPE specimens has to be abandoned. The probes for the targeted cancer gene sequencing were synthesized by BioEdge in May 2013. EdgeBio was later acquired by GeneDx before Jan 2014. GeneDx then discontinue the service for the Ion Torrent AmpliSeq sequencing in 2015.*

Owing to the low yield of DNA extracted from tumor specimens, targeted gene NGS, whole exome sequencing, LOH analysis or methylation analysis could not be completed without giving up some of the original plan. Given the quantity and quality of the extracted DNA, it is determined to undergo Affymetrix OncoScan FFPE® Assay for LOH and CN alteration using Molecular Inversion Probe (MIP) method designed for FFPE samples.

5b. *Collaboration with Dr. Gareth Evens from U.K. was canceled.*

5c. Data analysis and manuscript development for Aim 3. -- 50% completed

DNA extraction was applied to 11 specimens appearing to have enough tissue.

Initial plan for 31 genes **targeted next-generation sequencing (Ion Torrent AmpliSeq)** by EdgeBio (later acquired by GeneDx in December 2013) was abandoned because GeneDx discontinued such service. The Ion AmpliSeq custom primers were made by EdgeBio in May 2013.

Extracted DNA specimens were sent to the Moffitt Cancer Center (MCC) genomic core lab in June 2015 for next generation sequencing. The Ion AmpliSeq primer pool synthesized by GeneDx was also shipped to Moffitt Cancer Center.

It also became clear that there will not be enough DNA material for MLPA analysis to assess copy number and LOH.

After QC assessment of the DNA material, the quantity and quality of the DNA was much less than expected. In addition, Ion AmpliSeq was canceled owing to the uncertainty of its performance on FFPE DNA. It was determined that there was just enough DNA to undergo Affymetrix OncoScan FFPE® Assay for genomic copy number (CN) and loss of heterozygosity (LOH) analysis. OncoScan utilizes the highly specific molecular inversion probe (MIP) technology with 50-100 kb copy number resolution in ~900 cancer genes and 300 kb genome-wide copy number resolution outside of the cancer genes. It analyses the genome-wide allelic imbalances, detects copy number alterations, LOH, including copy neutral LOH from a single assay. It can accurately assess up to 10+ copy changes and has demonstrated

concordance with FISH-confirmed amplifications including. It has reported concordance with FISH-confirmed amplifications such ERBB2 (Her2), EGFR, MDM2, MYC, and FGFR1. The *NF1* gene region has a high density coverage of 65 probes.

OncoScan results showed satisfactory data quality on 7 samples. Suboptimal data quality were seen on certain segments of the chromosomes on 3 samples, leaving one sample with poor quality data to be excluded from further analysis. CN status of these tumors was compared with that of 1105 sporadic breast cancers from The Cancer Genome Atlas (TCGA) provisional data set.

Data analysis for OncoScan results is still ongoing. Preliminary findings include:

1. As described in the above section (Task 4), a significantly higher proportion of the specimens underwent OncoScan (6/10, 60%) showed positive staining for HER2/neu (Human epidermal growth factor receptor 2), in comparison to the 15-25% in sporadic breast cancer, p=0.02. In addition, OncoScan shows a significantly higher proportion of the specimens (5/10, 50%) with CN gain of *ERBB2*, the gene encoding HER2, comparing with 15% in sporadic tumors, p=0.01. No *ERBB2* CN loss is observed in this cohort in comparison with the 12% in sporadic tumors.

2. Sixty percent (6/10) of the samples contain LOH involving the entire *NF1* gene or at least ¾ of the gene. One sample with LOH and CN gain (CN=2.33). Two samples with LOH and clonal CN loss.

3. A segmental LOH on chromosome 16p11.2 is observed on 7/10 samples, encompassing 2.66 Mb, Chr16:32,608,732-35,271,725 (GRCh37/hg19). This region is covered by 80 probes. LOH in this region has not being reported in breast cancers. Known genes encoded in this region are: *UBE2MP1, FLJ26245, LINC00273, SLC6A10P, IGH, RNU6-76P, MIR-4518/4518, TP53TG3, TP53TG3B, TP53TG3C,* and *FRG2DP*.

4. Another segmental LOH on chromosome Xq11.1 is observed on 7/10 samples, encompassing 1.95 Mb, ChrX:63,036,457-64,987,692 (GRCh37/hg19), This region is covered by 150 probes. LOH in this region has not being reported in breast cancers. Known genes encoded in this region are *AMER1, ASB12, MTMR8, ZC4H2, LAS1L, MSN, ZC3H12B,* and *FRMD8P*1.

5. A significant number of LOH were observed on Chr 17p and the proximal portion of the Chr 17q. At least 3 samples exhibit clonal LOH of the entire Chr 17. According to the CN status in sporadic breast cancers, we do not observe unusual rate of CN loss for the genes located on the Chr 17, i.e. *TP53*, *MAP2K4*, or *BRCA1* genes.

Data analysis is still ongoing for OncoScan results.

Aim 4:
Phenotypic Analysis of NF1 knockdown in normal mammary epithelial cells

These cell line studies have been conducted in Dr. Michael Tainsky's laboratory in Wayne State University.

In order to study the association of Neurofibromatosis Type I to breast cancer development we are examining the effect of NF1 knockdown (KD) in human mammary epithelial cells (HMECs) derived from breast mammoplasty procedures. This study will provide information on the molecular mechanisms of breast cancer in NF1 deficient human subjects and will help to determine guidelines for the screening of NF1 breast cancer patients.

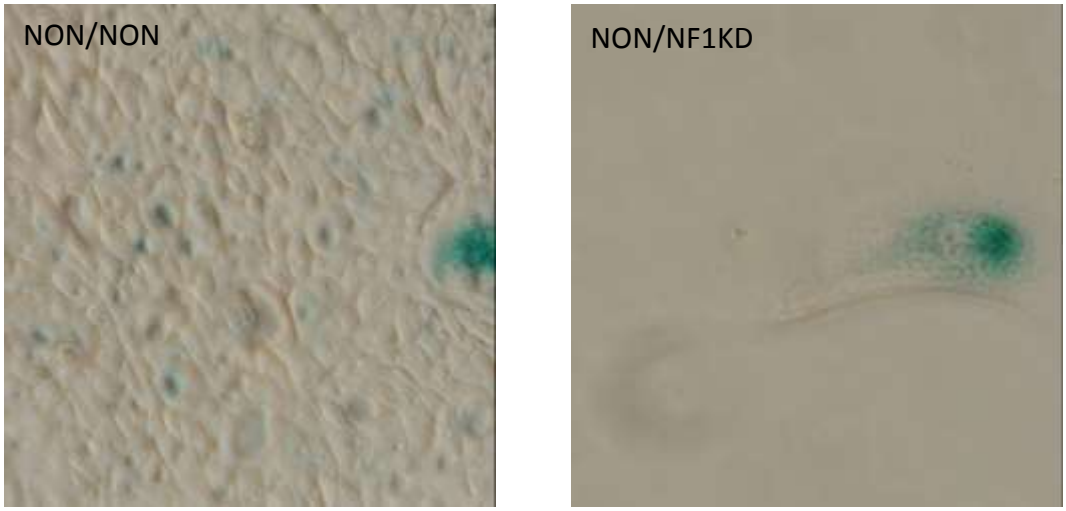
It has been shown that ablation of NF1 results in activation of Ras oncogene and that such activation results in transformation or senescence in different cell types. We performed experiments to determine whether these mechanisms could be induced in HMECs by an NF1 deficiency and the involvement of RB1 and p53 pathways.

NF1 knock out triggers cellular senescence in HME cells.

In order to study NF1 knock down in HMEC, we first had to establish conditions for the experiment using shRNAmir-NF1 (Openbiosystems, ThermoFisher Scientific). We created the lentiviral vector upon transfection of plasmids + shRNA into packaging cell line 293T, determined the titer of the virus and the infection efficiency. Each double infection was performed within 24 hours incubation from the first infection. The control cells were infected with the empty lentivirus (NON).

Same number of HME cells were plated before infection and a β-Gal assay was performed after 2 weeks of selection in puromycin. Fig. 1 shows the dramatic difference in cell number and phenotype between the control cells, NON/NON and the NF1 knockdown cells (NON/NF1 KD). Evidently the NF1 KD cells stop growing and undergo senescence. Senescent cells undergo phenotypic changes as they are enlarged and flattened and show increased B-Gal activity (blue cells).

Fig.1



Infections were repeated 3 times with slightly different conditions, but they always gave the same result: HMEC growth arrest and early senescence after depletion of NF1.

The NF1 knockdown was checked by western blot (Fig2) and the relative protein amounts were calculated in comparison with tubulin amounts (Graph 1). The decrease in NF1 expression is about 1/10 compared to the control.

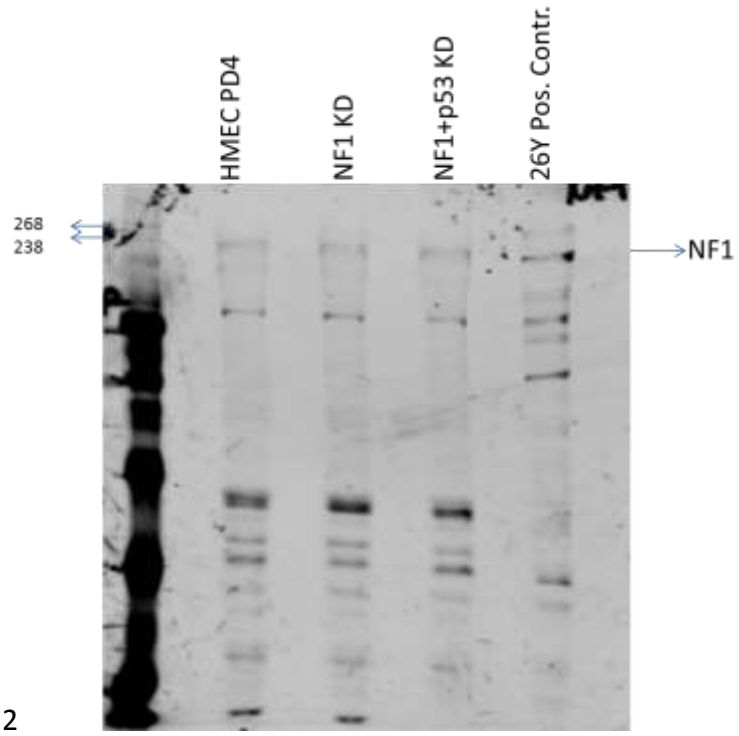
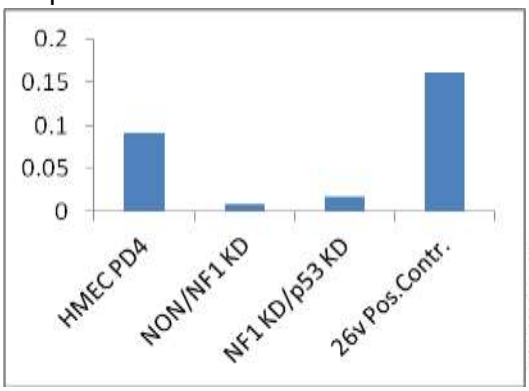


Fig.2

Graph 1



HMEC PD4	0.091048
NON/NF1 KD	0.008984
NF1 KD/p53 KD	0.017496
26v Pos.Contr.	0.16219

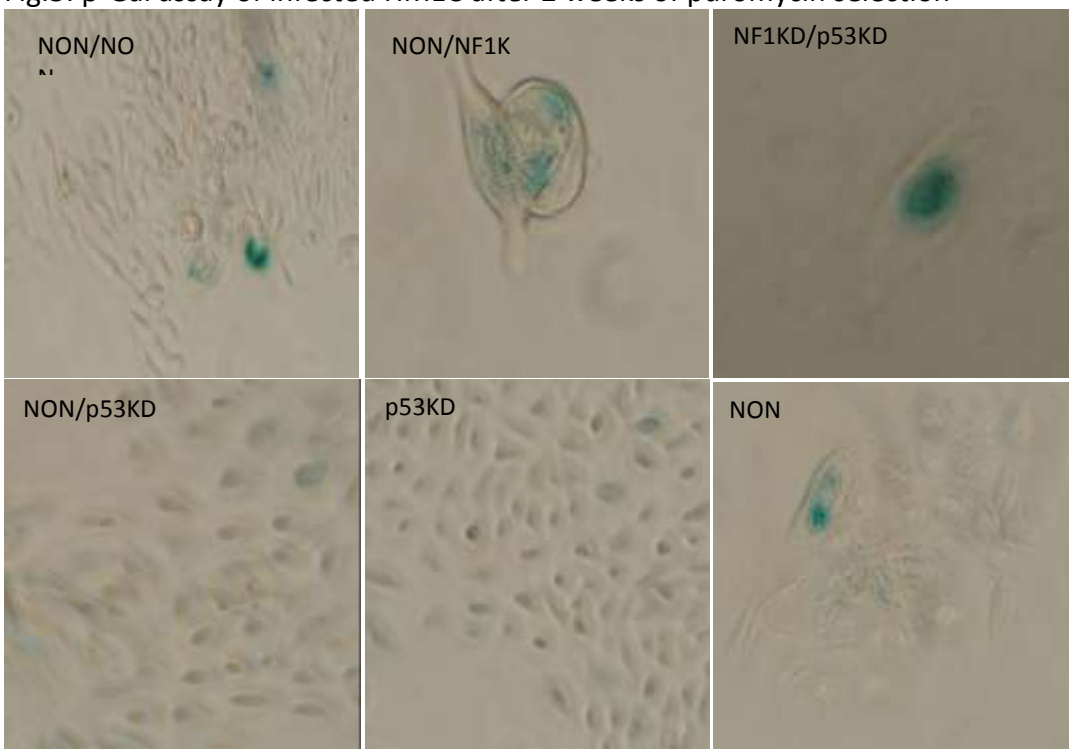
Loss of p53 or RB does not rescue senescent NF1 KD HMEC

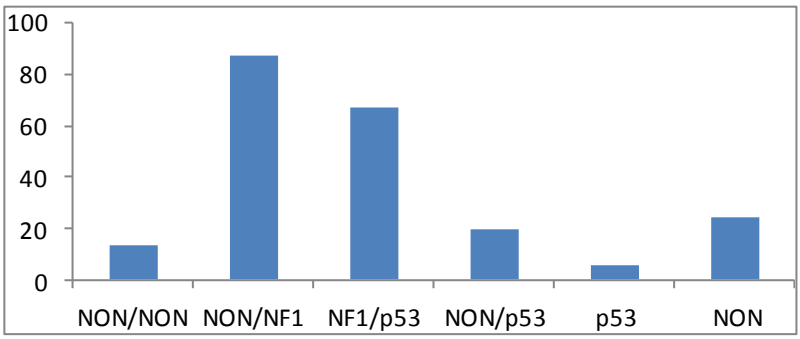
It is known that ablation of NF1 results in activation of Ras oncogene in mouse embryonic fibroblast (MEF) and such activation might trigger an oncogene induced senescence in cells (OIS). Known regulators of senescence are p53 and/or RB pathways, but other signals might be involved to suppress the oncogenic expression and induce senescence.

In order to understand the mechanisms that trigger senescence in HMEC after NF1 depletion and the involvement of p53 and/or RB in such process, we also prepared lentiviruses sh-RNA-p53 and sh-RNA-RB1.

The knock down of p53 alone in HMEC resulted in a temporary cell growth advantage for the first 2 weeks of culture when compared to the controls. However we observed an increased rate of cell death. After 4 weeks of cell culture, HMEC p53 KD became senescent and appeared not much different from the control cells (Fig.3), confirming data already published by Garbe et al, 2007. The double knockdown of p53 and NF1 in HMEC (NF1 KD/p53 KD) shows early senescence when compared to the HMEC control (NON/NON) and it is not able to rescue the cells from the senescent phenotype caused by NF1 knock down alone (Fig.3 and Graph2).

Fig.3: β-Gal assay of infected HMEC after 2 weeks of puromycin selection





Graph 2: Percentages of blue cells obtained after performing β-Gal Assay of infected cells

Interestingly the knockdown of RB1 in HMEC gave a growth advantage to NF1 KD HMEC for 14 days after performing the infections. Graph 3 shows the reduction in cell number after 7 days selection. HMEC NF1 KD infected with RB KD retained a higher number of cells compared to the NON/NF1 KD, suggesting a growth advantage and the prevention of senescence in the cells where RB is less expressed. Unfortunately when the cells were detached from the original plate and passed to new plates we observed a very high rate of cell death and the formation of few clones (Fig.4). However after a month in culture, the cells were not able to grow and the density and the phenotype of the cells were the same throughout different infections including the control (Fig.5). We grew the cells for an additional 3 weeks without signs of cell growth in any infections.

Graph 3

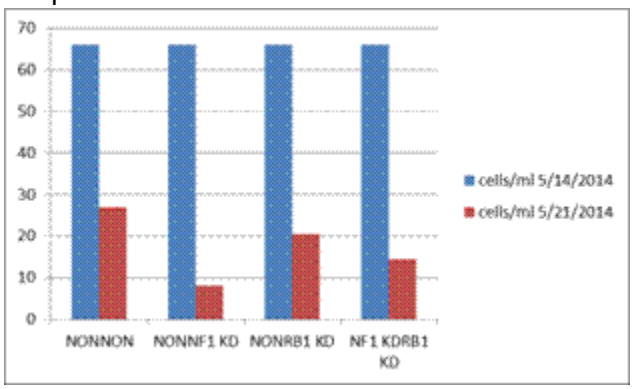
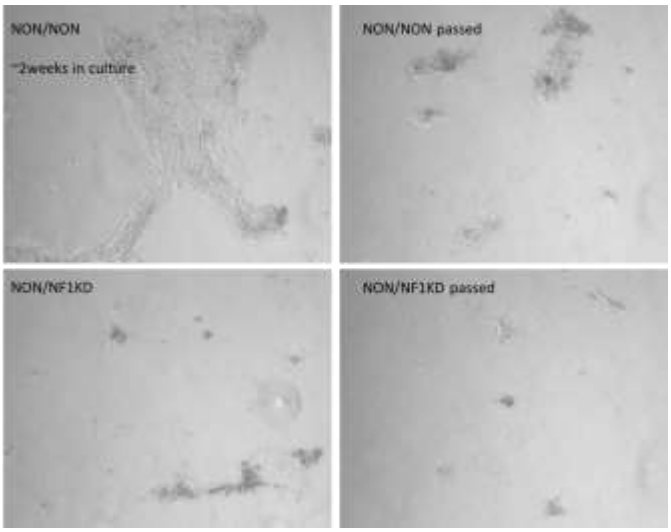


Fig. 4



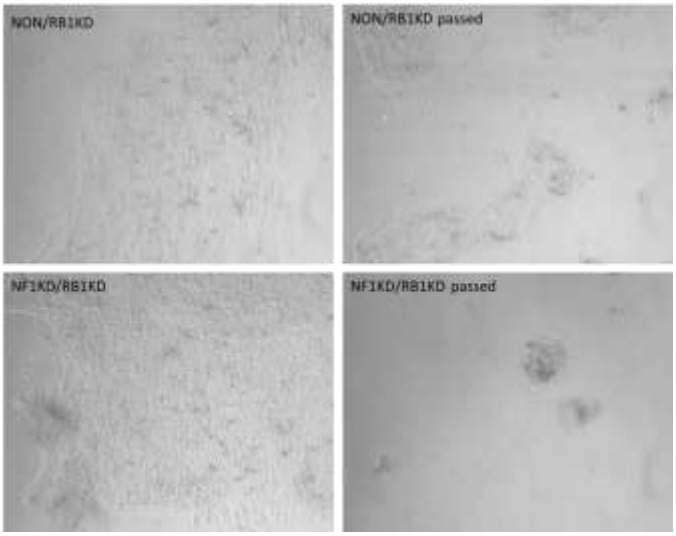
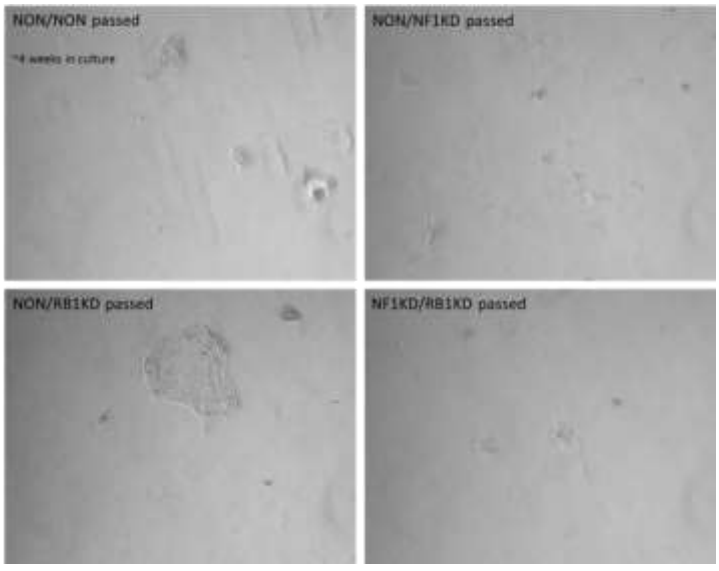


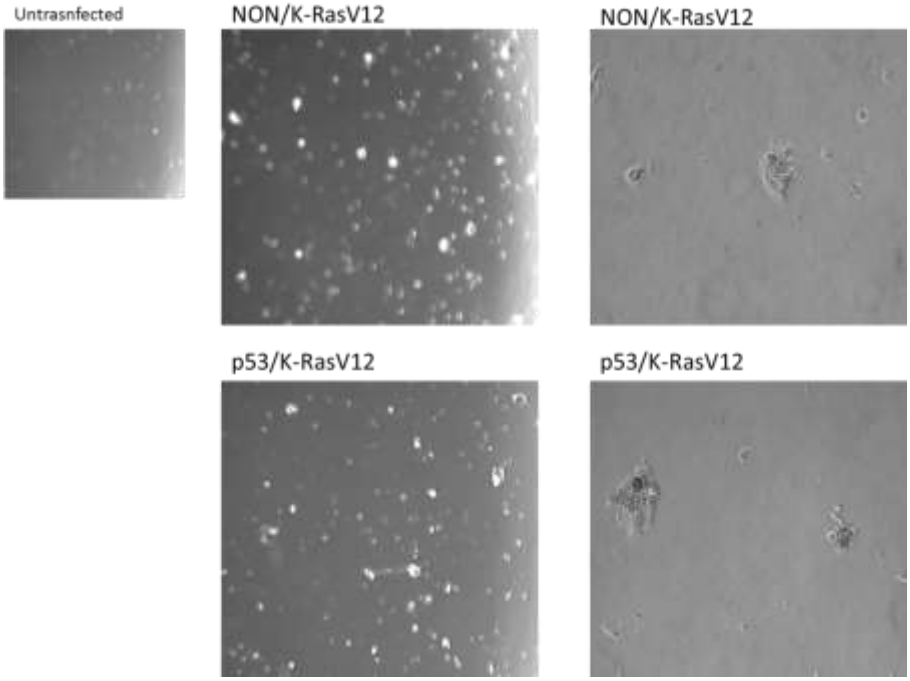
Fig. 5



Overexpression of mutant Ras does not result in immortalization /transformation of HMEC

It has been shown that MCF10A escape OIS when transfected with mutated Ras(V12) by degrading C/EBPbeta1 and the cells become transformed. To assess the sensitivity of HMEC cells to mutated Ras, Ras (V12) was overexpressed in HMEC NON and HMEC p53KD by plasmid transfection using the X-tremeGENE 9 DNA transfection reagent (Roche). Even in the cells where p53 expression was reduced, KRas (V12) overexpression did not give a growth advantage to the cells (Fig.6).

Fig.6



Conclusions

- NF1 inactivation results in HMEC senescence
- p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC.

  p53 inactivation provides an initial growth advantage to HMEC with a consequent large number of cell death confirming data already published (Garbe et al 2007)
- RBKD HMEC show higher growth rate and a decreased cell death compared to p53KD HMEC

- Overexpression of K-Ras V12 does not transform p53 inactivated HMEC

Plans

NF1KD, P53KD and RBKD will need to be confirmed by Western and/or RT-PCR. Low levels of p53 and RB inactivation might be an explanation to the fact that we are not able to block the senescent phenotype of HMEC NF1KD.

Ras and Ras effector activity will need to be confirmed in HMEC cells and HMEC infected cells as well.

Further studies are needed to understand which of Ras negative regulators might be also involved in the OIS of HMEC NF1KD. It might be worth to study the process of HMEC induced senescence in the immortalized cell line, such as hTERT-HMEC.

- **What opportunities for training and professional development has the project provided?**
  Nothing to Report

- **How were the results disseminated to communities of interest?**
  Nothing to Report

- **How were the results disseminated to communities of interest?**
  Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**
  Nothing to Report

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

A. The following findings are likely to be used to guide targeted cancer screening for individuals (especially females) affected with NF1.

   a. The family history of breast cancer or all cancers may predict the personal risk of breast cancer in women with NF1.
   b. Plexiform neurofibroma may be a predictor for MPNST.
   c. Learning disability and European ancestry may be a predictor for central nervous system tumor, including optic glioma.

B. At this time, this study has not support the hypothesis that NF1 gene mutation is an independent hereditary risk factor for breast cancer. The moderately elevated risk of breast cancer in women with NF1 is likely a manifestation of the synergistic effects between the NF1 gene mutation, environmental carcinogens and/or other hereditary cancer predisposition genomic variants. When exists independently, these variants are likely of moderate or low risk for cancer. Result analysis from OncoScan has not been fully completed yet.

- **What was the impact on other disciplines?** Nothing to Report
- **What was the impact on technology transfer?** Nothing to Report.
- **What was the impact on society beyond science and technology?** Nothing to Report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change (**all of the major changes have received approval from DOD and IRB**)**

1. One major change in this project is that in December, 2014, Dr. Xia Wang, the principle investigator who designed and initiated this project, left HFHS in Detroit, Michigan and moved to Moffitt Cancer Center in Tampa, Florida. The PI is transferred to Dr. Dhananjay Chitale, one of the major collaborators in HFHS. With the consent and collaboration from HFHS and Dr. Chitale, Dr. Wang continued to manage this project till the end.

2. Within the time period of this project, the rapid speed of DNA sequencing technology development and the change of its availability has enabled us to adopt newer technologies that are much more comprehensive than the ones previously planned.

3. For Aim 2, germline whole exome sequencing (WES) was added for NF1 women with a history of breast cancer (This plan was added in July, 2013). Due to the significant artifacts and false positive results from WES on the test cases, WES on 42 control germline lymphocytes DNA samples was added in Feb 2015).

4. Plan to collaborate with Dr. Gareth Evens from U.K. to collect more tumor specimens did not work out. Dr. Evens declined the initial offer. This plan was cancelled in the spring of 2014.

5. For Aim 3 Task 4, limited amount of tumor tissue as well as the DNA extracted from the tumor tissue forced us to abandon the plan for loss of heterozygosity (LOH) and methylation analysis by MLPA method. We have also abandoned the plan for Agilent OneSeq to accomplish tumor sequencing and copy number variation/LOH analysis and abandoned the plan to use Illumina 450K array for methylation analysis.

6. LOH assay was changed to Affymetrix OncoScan FFPE® Assay which assesses copy number alteration and LOH. This plan of change was made in November 2015.

7. For IHC assay on tumor specimens, all protein IHC was completed except Ki-67. No control samples were done. IHC was not done on control samples as originally planned. The reason is: 1) The case number was too small and findings were too un-representative to generate any statistical significance, even in the presence of control samples. Ki-67 IHC was not done due to lack of tissue and manpower.

8. For Aim 3 Task 5, initial plan for 31 genes **targeted next-generation sequencing (Ion Torrent AmpliSeq)** by EdgeBio (later acquired by GeneDx in December 2013) was abandoned because GeneDx discontinued such service. The Ion AmpliSeq custom primers were made by EdgeBio in May 2013. Extracted DNA specimens were sent to the Moffitt Cancer Center (MCC) genomic core lab in June 2015 for next generation sequencing. The Ion AmpliSeq custom primer pool synthesized by GeneDx was also shipped to Moffitt Cancer Center. After QC assessment of the DNA material, the quantity and quality of the DNA was much less than expected. In addition, the performance of Ion AmpliSeq on FFPE DNA remains to be uncertain. It was determined that there was just enough DNA to undergo Affymetrix OncoScan FFPE® Assay for genomic copy number (CN) and loss of heterozygosity (LOH) analysis. This change of plan was made in November 2015.

There is no significant impact on the expenditures. All technological changes are planned without needing extra fund.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: None
- **Significant changes in use or care of human subjects:** None
- **Significant changes in use or care of vertebrate animals:** Does not apply**.**
- **Significant changes in use of biohazards and/or select agents:** Does not apply.

6. PRODUCTS

- **Publications, conference papers, and presentations:**

  The content and results from Aim 1 are reported in a manuscript. **The manuscript has been accepted by the Journal of Genetic Syndromes and Gene Therapy in March 2016**.

  Poster presentation related to Aim 1:

  1. 6-2013 CTF (Children's Tumor Foundation) annual conference "The Incidence of Cancer in Women with Neurofibromatosis Type 1" Renée Tousignant, MS, MSC, Xia Wang, MD, PhD, FACMG & Albert Levin, PhD
  2. 6-2014 CTF annual conference "The Incidence of Neoplasms in 424 Women with Neurofibromatosis Type 1" Xia Wang, MD, PhD, Renee Tousignant, MS, CGC *Henry Ford Health Group*; Albert Levin, PhD, Henry *Ford Health System*; Bruce Korf, MD, PhD, *University of Alabama at Birmingham*; Jaishri Blakeley, MD, *Johns Hopkins University*; Maria Acosta, MD, *Children's National Medical Center*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Xia Wang MD, PhD*
Project Role:	*Consultant, previous PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2 *4 years*
Contribution to Project:	Dr. Wang has been organizing and directing the entire project process towards its completion. She has been responsible for the reporting, communication, budgeting, securing the labs and personnel, and writing the manuscripts for publication.
Funding Support:	*Currently employed in Moffitt Cancer Center, previously employed in Henry Ford Health System; supported by DoD*

Name:	*Dhananjay Chitale, MD, PhD*
Project Role:	*Current PI, co-investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1 *2 years*
Contribution to Project:	Dr. Chitale is the official PI for this reporting period. He is aware and has approved Dr. Wang's management for this project. He has also completed the micro-dissection of the tumor FFPE specimens, directed the IHC analysis for the proteins, and directed DNA extraction from tumor.
Funding Support:	*Employed in Henry Ford Hospital, supported by DOD*

Name:	**Brandon Shaw PhD**
Project Role:	Sequence analyzer
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1 * 1 year*
Contribution to Project:	Dr. Shaw participated in the preliminary analysis of germline whole exome sequencing (WES) data from NF1 women affected with breast cancer. He used Omicia genome sequencing data analysis program.
Funding Support:	*Employed by HFHS; supported by NF Michigan, a patient advocate organization*

Name:	**Renee Tousignant, MS**
Project Role:	Project coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.2 *2 years
Contribution to Project:	Data/sample collection in HFHS site, document preparation, obtaining consents from participants, document and data management, data analysis, coordinated data/sample collection from subaward sites, coordinated communication and information distribution among subaward sites.
Funding Support:	Was employed by Henry Ford Health System during the study time before July 2014; Supported by DOD
Name:	**Lisa Whitely**
Project Role:	Senior technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1* 1 year
Contribution to Project:	Tumor DNA extraction, DNA QC and storage
Funding Support:	Employed by HFHS: supported by DOD
Name:	**Kathy Roszka**
Project Role:	Senior technician
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	1* 1 year
Contribution to Project:	Tumor processing for IHC staining
Funding Support:	Employed by HFHS: supported by DOD
Name:	**Albert Levin PhD**
Project Role:	Statistician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6 *2 years
Contribution to Project:	Statistical analysis during the project planning phase and clinical data analysis phase; contribution to the direction of analysis.
Funding Support:	Currently employed by HFHS; supported by DOD
Name:	**Maria T. Acosta MD**
Project Role:	Co-PI of the subaward site
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5 *3 years
Contribution to Project:	Responsible for overseeing the project in the subaward site; ensure IRB, policy compliance, oversee the data collection, transmission, recruiting and consenting; participate in manuscript development
Funding Support:	Employed by Children's National Medical Center (CNMC); supported by DOD
Name:	**Debroah Copenheaver** MS
Project Role:	Research coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5 * 2 years
Contribution to Project:	Collect data; consent research participants; help to communicate with the HFHS site
Funding Support:	Was employed by CNMC during the study period; supported by DOD

Name:	**Jaishri Blakeley MD**
Project Role:	Co-PI of the subaward site
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5 * 3 years
Contribution to Project:	Responsible for overseeing the project in the subaward site; ensure IRB, policy compliance, oversee the data collection, transmission, recruiting and consenting; participate in manuscript development.
Funding Support:	Employed by JHU; supported by DOD
Name:	**Amanda Bergner MS**
Project Role:	Research coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5 * 3 years
Contribution to Project:	Collect data; consent research participants; help to communicate with the HFHS site
Funding Support:	Employed by JHU; supported by DOD
Name:	**Bruce Korf MD, PhD**
Project Role:	Co-PI of the subaward site
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5 * 3 years
Contribution to Project:	Responsible for overseeing the project in the subaward site; ensure IRB, policy compliance, oversee the data collection, transmission, recruiting and consenting; participate in manuscript development.
Funding Support:	Employed by UAB; supported by DOD
Name:	**Raven Winfrey MS**
Project Role:	Research coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5 * 2 years

Contribution to Project:	Collect data; consent research participants; help to communicate with the HFHS site
Funding Support:	Employed by UAB; supported by DOD
Name:	**Michael A. Tainsky PhD**
Project Role:	Co-PI of subaward site
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6 * 2 years
Contribution to Project:	Oversee the project; help to create and design this subaward project
Funding Support:	Employed by WSU; supported by DOD
Name:	**Kraniak, Janice PhD**
Project Role:	Research associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4 *1.5 years
Contribution to Project:	Help to create and design this subaward project; carry out the bench work for this subaward project
Funding Support:	Employed by WSU; supported by DOD

- **What other organizations were involved as partners?**

Moffitt Cancer Center (Tampa, Florida) has allowed Dr. Wang to use the time, computer equipment and facility to continue the project until completion.

A Summary of all the prior and current collaborating and contracted institutions and personnel is list as below:

Primary study site (HFHS – Henry Ford Health System):

Henry Ford Hospital, Pathology, 2799 W. Grand Blvd., K6, Detroit, Michigan 48202
Dhananjay Chitale, MD PhD, Current PI, previous Co-Investigator

Henry Ford Health Systems, Biostatistics and Research Epidemiology, One Ford Place, Place 5C, Detroit, Michigan 48202
Statistical analysis
Next generation sequencing and WES manual analysis
Albert Levin, PhD, collaborator

Moffitt Cancer Center, Genomics and Individualized Cancer Management, 10920 McKinley Drive, Office 5101, Tampa, Florida 33612
Xia Wang, MD PhD, Current consultant, previous PI

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Facility for tumor ion-torrent gene sequencing: We have initially contracted with EdgeBio for this task. EdgeBio synthesized the primers for target gene tumor sequencing. However, EgdeBio later was acquired by GeneDx. Then GeneDx discontinued providing this service.

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8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

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Indicator Exploration for Cancers in Women with Neurofibromatosis Type 1 – A Multi-center Retrospective Study

Running head: The cancers in women with NF1

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a pleiotropic autosomal dominant hereditary syndrome. It is characterized by various types and numbers of benign and malignant neoplasms. The occurrence of gliomas, malignant peripheral nerve sheath tumors (MPNSTs), gastrointestinal stromal tumors (GISTs), and pheochromocytomas is significantly elevated compared to the general population.[1,2] The rate of colon and breast cancers are moderately increased, especially among individuals 50 years or younger.[3-7] A hospital admission based record-linkage population study has also shown an elevated risk for other common cancers, such as liver, esophagus, stomach, pancreas, biliary tract, lung, skin, thyroid, ovarian, leukemia and lymphoma in people with NF1.[8] The spectrum of non-neoplastic clinical and physical features of NF1 is also wide. Despite the increased risk for malignant neoplasms, there is no established protocol to screen for cancer in people with NF1 beyond the guidelines for the general population. If any clinical features of NF1 and/or family history are found to be associated with occurrence of certain

neoplasms, these may serve as indicators for targeted cancer or neoplasm surveillance. This multi-center case review study was designed to explore the associations between the occurrence of neoplasms and the physical/clinical features of NF1 in women with NF1. The overall goal is to identify factors associated with breast cancer in women with NF1.

MATERIALS AND METHODS

Study Subjects

Comprehensive medical record review was conducted in three Children's Tumor Foundation (CTF) affiliated neurofibromatosis clinics in the United States. These include Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), and Johns Hopkins University (JHU). Children's National Medical Center (CNMC) in the District of Columbia also recruited and collected medical information from affected mothers whose children were evaluated in the NF clinic. The medical records were reviewed for all females 20 years or older at the time of study, who either meet the consensus clinical diagnostic criteria of NF1 [9] or carry a deleterious mutation in the *NF1* gene. The four hundred and twenty three cases collected include all women who were seen in the clinic during the following periods of time: 114 cases (1994 to 2013) in HFHS, 122 cases (2011 to 2013) in UAB, and 156 cases (2003 to 2013) in JHU. In CNMC, 31 cases were collected from 2011 to 2013.

Data Collection

Demographic information gathered included date of birth, ethnicity, and biological relationships within the cohort. Medical information gathered included clinical features, such as the number of café-au- lait macules on the skin, presence of skin fold freckling, Lisch nodules on the irises, bony dysplasia,

macrocephaly, short stature and learning disability. Neoplasm-specific information collected includes the number of cutaneous neurofibromas, plexiform neurofibromas (PN), optic gliomas (OPG), malignant peripheral nerve sheath tumor (MPNST), as well as other malignant solid tumors, malignant hematological disorders, malignant or benign tumor of the central nervous system (CNS). OPG is a tumor originated from neural glial astrocytes. It develops on the tract of optic nerve during the first several years in life. In this report, it is discussed as a separate entity from other CNS tumors.

For women identified as having breast cancer, the histological type, stage and age at diagnosis were recorded when available. Breast cancer screening and breast biopsy information was also collected. Family history information gathered included NF1, malignant neoplasm, CNS tumor, and the number of relatives with breast cancer based on three-generation pedigree obtained by a genetic counselor. Genetic test results such as *NF1* gene mutation and/or *BRCA1* and *BRCA2* mutation were documented when available. The occurrence of malignant neoplasms and CNS benign or malignant tumors were assessed for their possible association with clinical features associated with NF1. The CNS tumor category includes all tumors, from low grade glioma to high grade glioblastoma. A feature of thickened optic nerve or chiasm was not counted as OPG. The source of information and clinical features documented in the medical record was either self-reported by the patients or supported by clinical evidence.

Statistical Analysis

While an attempt was made to collect complete data on all subjects, the validity of multivariate analysis was limited due to missing data. Therefore, we have restricted the presentation of results to only those from the univariate analyses, assuming that the data for individual variables are missing completely at random. We used Fisher's exact tests to evaluate the statistical significance of association between each

discrete clinical feature and prevalent cancers. P-values less than 0.05 were considered to be statistically significant. For ease of interpretability, odds ratios (OR) and corresponding 95% confidence intervals were also estimated to provide estimates of effect.

This research project has been prospectively reviewed and approved by the Institutional Review Board (IRB) of each participating center and by the Human Research Protection Office (HRPO) of the U.S. Army Medical Research and Material Command.

RESULTS

A total of 423 cases of women affected with NF1 were reviewed. Average age for this cohort is 40±14.7 years. Median age is 38 years. The study sample comprised 250 European Americans, 118 African Americans, and 41 individuals of other ethnicities. Ethnicity information was not available for 14 women. Thirty-six women are related to at least one other woman in this cohort and belong to a total of 16 kinships (Table 1). Family history of NF1 in female relatives was collected based on the pedigree in the medical chart. At least one female relative was affected with NF1 for 162 women. There were no female relatives affected with NF1 for 215 women. The status of family history of NF1 was not available for 46 women.

At least one type of cancer was reported in 98 women with NF1. Nineteen of them have had at least two primary cancers. The breakdown of observed neoplasms is presented in Table 2. There were 205 prevalent cancer/neoplasms in the relatives of 125 women with NF1. These included 9 NF1 related cancers (consisting of brain tumor and MPNST), 4 neuroendocrine tumors (consisting of pheochromocytoma and pituitary tumor), 4 sarcomas, 8 hematological cancers, 75 breast cancer, and

105 other cancers (consisting of 21 lung, 18 colorectal, 4 esophageal, 2 gastric, 6 head and neck, 3 cervical, 5 ovarian, 3 uterine, 3 bladder, 12 prostate, 3 renal, 9 skin or melanoma, 3 pancreatic, 3 thyroid, 6 “bone”, 2 “thoracic” cancer and 1 metanephric stromal tumor).

Breast cancer: Of the 20 women who have a personal history of breast cancer, 15 were previously reported by Wang [5] and Madanikia [6] in 2012. Eleven are European Americans and 8 are African Americans. Ethnicity information for the remaining individual is not available (Table 3). None of these women are known to be genetically related to one another. Half of the cases (n=10) were diagnosed with breast cancer between the age of 40 to 49 years. A quarter of the cases (n=5) were diagnosed between 30 to 39 years of age. Two cases were diagnosed with a second primary breast cancer. All of these breast cancers are ductal carcinoma, except one invasive lobular carcinoma, which is estrogen receptor (ER) positive (Table 4). Only one case was known to be an ER-PR (estrogen-progesterone receptor) negative and HER2 expression negative (i.e. triple negative) invasive ductal carcinoma. Two cases are known to be ER-negative, HER2 expression positive tumors.

Breast cancer and family history: The prevalence of personal history of breast cancer was nearly four-fold higher (odds ratio OR=3.83, 95% confidence interval 95%CI=1.40-11.12) for women with NF1 and a family history of any cancers (9.6%; 12/125) in comparison to those without a family history (2.7%, 8/298), which was statistically significant (p=0.004). The type of cancers in the family history does not differ significantly between the women with breast cancer and those without. However, when there is a family history of 3 or more cancers, the rate of personal breast cancer is 4 times higher (26.3% 5/19) than the rate when there are only 1 or 2 cancers in the family (6.6% 7/106), p=0.019. The prevalence of

personal breast cancer with a family history of breast cancer in 1st, 2nd, and 3rd degree female relatives (10.7%, 8/75) is more than 3-fold higher (OR=3.46; 95%CI=1.09 – 11.02) than without a family history (3.3%, 8/241), which is statistically significant (p=0.029). However, breast cancer is not significantly associated with family history of female relatives with NF1 (p=0.434). In this cohort, none of the women with breast cancer had a reported family history of any relative affected with NF1 and breast cancer.

Breast Cancer and Clinical Features of NF1: For cases with available clinical features, statistical analysis has not detected any association between the NF1 features, breast cancer or other cancers (all p≥0.16, Supplementary table 1). It is noteworthy to mention that high cutaneous neurofibroma burden (20 or more or described as "diffuse" in the medical record) is not significantly associated with any types of cancer (p=1.00).

MPNST and Plexiform Neurofibroma: The occurrence of MPNST is related to plexiform neurofibromas (PN). Among women with documented PN, 7.9% (11/139) have a history of MPNST, which is significantly higher (p=0.049) than the women without, 3.14% (7/223).

CNS tumor, optic glioma (OPG) and learning disability: The prevalence of CNS tumors is significantly higher (p=0.004) in women with a history of OPG (14.6%, 6/41) in comparison to women without OPG (2.9%, 8/278). The women with learning disability have a 2.25-fold (95%CI=1.08-4.67) higher rate of CNS tumor, OPG or both (i.e. "CNS+OPG") (22.2%, 20/90) than those without a learning disability (11.2%, 21/187), which is significant (p= 0.019). Due to the small number of cases, the

relationship between learning disability and CNS tumor excluding OPG cannot be determined at this time. However, upon exclusion of the cases with CNS tumor alone, the association between learning disability and OPG with or without CNS tumors is suggestive, but not statistically significant in this cohort, 16/90 vs. 19/187, p=0.083.

Ethnicity and Malignant Neoplasms: The rate of "CNS+OPG" and "Other cancers" varies significantly by ethnicity. "Other cancers" refers to all malignant tumors, hematological malignancies, CNS tumors and OPG, excluding breast cancer. For the "CNS+OPG" category, European Americans (EAs) were 3.72 times (95% CI=1.48 – 11.16) more likely to develop these tumors (21.2%, 41/193) than African Americans (AAs) (6.8%, 6/88), which was statistically significant (p=0.002). The occurrence of OPG with or without CNS tumor is also higher (OR=3.48, 95%CI=1.28 – 11.88, 95%) in EAs (17.4%, 32/184) than AAs (5.7%, 5/88), which was significant (p=0.008), For the "Other cancers" category, EAs were also significantly (p=0.004) more likely to develop these tumors (26.8%, 67/250) than AAs (13.5%, 16/118). Analysis could not demonstrate a statistically significant association between ethnicity and breast cancer (p=0.301).

Ethnicity and other clinical features: Lisch nodules are more common in EAs (59%, 100/170) relative to AAs (39%; p=0.009, 26/66) or other ethnicities (32%; p=0.010, 10/31). There is also a significant difference between the number of individuals with higher dermal neurofibroma burden, i.e. 20 or more or described as "diffuse" at the time of clinical evaluation, by ethnicity, with AAs having a higher rate (75.8%, 47/62) of high tumor burden than EAs (53.0%, 70/132; p=0.003).

DISCUSSION

The multi-systemic involvement of NF1 and apparent physical signs has had inspired studies to investigate the association between these signs to shed light on the underlying molecular mechanisms. [10, 11] The current study aimed at finding the association between physical signs, clinical features, family histories and malignant neoplasms. The strength of this study is that it is a multi-center study representing an adult NF1 patient population from widespread geographical areas within the United States (Baltimore and Washington D.C. on the east coast, Detroit in the Mid-West and Birmingham of Alabama in the South). The percentage of African Americans in this study is higher relative to most other studies, thus providing a novel insight into the clinical profile of NF1 in this population. The weakness of the study is that it is a retrospective study with its associated biases. Data regarding screening, detection and treatment of cancers other than breast, was not collected. Additionally, the study cohort represents individuals with NF1 seeking care in a large academic and/or tertiary care center in adulthood, likely with relatively severe disease manifestations or morbidity. Family history recall by patients may be biased by personal health situations occurring at the time the pedigree was obtained. In addition, only female cases were analyzed.

Previous studies have revealed a significantly elevated breast cancer risk, 4-8 fold, in women with NF1 under age 50 in England and the United States.[3-7] For women age 50 or older, the risk is also elevated, but to a lesser degree, 1.9-2.6 fold. This phenomenon leads to the suspicion that a pathogenic germline *NF1* genetic variant may be an independent risk factor for breast cancer. Based on this assumption, family history of NF1 should be associated with breast cancer in this population. However, this hypothesis is not supported by the results of this study. This study demonstrates that a personal history of breast cancer in women with NF1 is associated with a family history of breast cancer (OR=3.46) or

all cancers (OR=3.83) but does not appear to be associated with family history of NF1 alone in female relatives. Nevertheless, we cannot exclude the possibility that the association between personal history of breast cancer and family history of breast cancer is enhanced by family history recollection bias. It is possible that patients who had a personal history of cancer were more likely to report a family history of cancers during the pedigree collection. Additionally, we cannot exclude the possibility that the lack of association between personal history of breast cancer and family history of NF1 may be partially due to the following two factors: 1) The study may lack sufficient statistical power due to the small sample size. 2) Breast cancer may not have manifested itself yet in some of the female relatives with NF1. The ages of relatives with NF1 were not collected therefore the percentage of relatives under age 30 is unknown. Nevertheless, a general population study utilizing a Swedish database has previously characterized the elevated breast cancer risks in association with family history of breast cancer in first and second degree relatives. The relative risk (RR) was 1.27 when a sister was affected. The RR was 1.74 when a mother was affected. The RR was 2.8 when at least 2 first degree female relatives were affected.[12] This association appears to be at a lesser degree than was observed in our cohort of NF1, however, the family histories in our study included all first, second and third degree relatives and were not stratified based on the degree of relationship. It is unclear at this time whether the higher prevalence of breast cancer in NF1 is a result of a dysfunctional *NF1* gene, environmental carcinogens and/or other hereditary cancer predisposition genomic variants or a synergistic effect between these three factors. The pattern of non-breast cancers in the family history offers no clue as what sort of carcinogens or hereditary genomic predispositions may be involved. No germline mutations in hereditary high penetrance breast cancer genes, *BRCA1* or *BRCA2,* have been reported in the 20 cases of breast cancer. Exploration of the co-occurring germline genomic mutations or variants may provide further clues. Germline Whole Exome Sequencing (WES) in a series of 14 NF1 women affected with breast cancer

has been completed by Wang and colleagues. Five of the cases are from the current study. Preliminary analysis showed no deleterious mutations in *BRCA1, BRCA2, TP53, PTEN, CDH1, PALB2,STK11* or *PALB2* genes. Based on the current data, the use of family history of breast cancer or any cancers as a risk indicator for personal breast cancer in women affected with NF1 may be a valuable tool.

The distribution of the histological types and hormonal receptor status for the breast cancers in women with NF1 does not differ significantly from the general population, except ER-negative tumors are under represented. Another manuscript will explore this in detail.

Whenever there is a heavy cutaneous neurofibroma burden, dermal neurofibromas on the breasts may be seen. Multiple bilateral dermal neurofibromas may be categorized as benign on mammography, with the relevant clinical history. Neurofibromas within the breast parenchyma are also common and may present as a new mammographic mass or a newly palpable finding on physical examination by the patient or health care provider. For neurofibroma within the parenchyma, physical examination may not reliably distinguish it from a primary breast malignancy. Based on the current standard of care, palpable findings in women over the age of 30 should be evaluated with mammography and ultrasound. On mammography and ultrasound, neurofibromas within the breast commonly present as a non-calcified solid mass with a round or oval shape and circumscribed or obscured margins. If deemed probably benign based on imaging criteria, neurofibromas within the breast may require subsequent follow up imaging. Similar to mammography and ultrasound, limited data exist regarding reliable differentiation of neurofibromas from invasive breast cancers based upon morphology and enhancement kinetics on MRI. However, for a neurofibroma with a myxoid matrix, the high T2 signal intensity of neurofibromas is a classic feature in the correct clinical setting.[13] Whether or not breast MRI may be valuable to reduce biopsies of palpable neurofibromas or as a supplemental screening modality in women with NF remains to be explored.

The association between MPNST and plexiform neurofibroma supports the previous evidence that the majority of MPNSTs emerge from preexisting plexiform neurofibroma.[1,14] Therefore, preexisting PN may also serve as a risk indicator for MPNST.

The current study suggests that OPG during childhood may serve as a risk indicator for future occurrence of brain tumor in individuals with NF1. The association between OPG and brain tumor has

been reported by Singhal and colleagues where 17 cases of NF1 related OPG were followed prospectively [15]. However, our study was not designed to collect the timing of diagnosis or the character or treatment of OPG. Asymptomatic OPG or other low grade CNS glioma without progression or a need for treatment may have been an incidental finding when brain was imaged for other reasons. Therefore, we cannot exclude the possibility that at least two factors have partially contributed to the association: 1) Asymptomatic OPGs were discovered during other CNS tumor evaluation, or vice versa; 2) Radiation therapy for OPG induced the CNS tumor later in life. An increased rate of CNS tumors later in life was previously reported among patients who have had radiation therapy for OPG.[15,16] The current standard is to avoid using radiation therapy for OPG in individuals with NF1.

Our study demonstrated an association between learning disability and CNS and/or OPG tumor, an observation also reported previously.[15,17] This association suggests a common defect hindering the CNS development congenitally as well as predisposing to CNS tumor formation later in life. In individuals with NF1, OPG mostly occurs during early childhood. As treatment for OPG, chemotherapy or radiation is known to have adverse effects on the developing brain, leading to learning disability. In a recent 20 year-perspective study of OPG, 149 children were diagnosed with OPG by MRI screening. Only 22 children required treatment.[18] Nevertheless, learning disability as a side effect of OPG treatment and/or a large tumor altering brain function could have partially contributed to the association between these two variables. More advanced study with information regarding OPG treatment, as well as metrics assessing learning disability before and after CNS or OPG treatment will allow us to better characterize the relationships.

Our study shows that predisposition to CNS tumors and/or OPG is disproportionally higher in European Americans, in comparison to African Americans. Although the observation in this study could be compounded by the possible unequal access to brain imaging between races, we do not believe the higher frequency of tumor in EAs is entirely resulted from easier access. A prior report of smaller sample size in 1998 suggested a similar predilection for OPG in EAs versus AAs.[19] A recent larger cohort retrospective study has also demonstrated this phenomenon.[18] This trend coincides with the observations that the incidence of sporadic malignant CNS tumor in non-Hispanic whites is around 2-4 times that of blacks in North America.[20] All of above suggests that the CNS tumor or OPG in NF1 patients may share a common pathway in tumorigenesis as sporadic brain tumors. As such, European American ethnicity may be a risk indicator for brain tumor in the population with NF1. However, in the absence of additional data, including grade, progression, and the need for treatment in these brain tumors, the life-time risk and the need for screening cannot be adequately evaluated.

Table 1 Demographic distribution of all subjects

Age Group	European American	African American	Others	Not available	E:A Ratio
All	250	118	41	14	2.12
20-29	71	35			2.03
30-39	59	29			2.03
40-49	48	21			2.29
50+	72	33			2.18

E:A ratio: European American to African American ratio

Table 2 Distribution of neoplasms in women with NF1

	Patients (n=423)	%
CNS tumor	18	4.3%
OPG	41	9.7%
CNS tumor and OPG	5	1.2%
MPNST	22	5.2%
GIST	6	1.4%
Breast	20	4.7%
Other Neoplasms	13	3.1%
Plexiform Neurofibroma	142	33.6%

CNS: Central Nervous System
OPG: Optic Glioma
MPNST: Malignant Peripheral Nerve Sheath Tumor
GIST: Gastrointestinal Stromal Tumor

Table 3. Family history and age at the diagnosis of breast cancer.

ID	Age at Diagnosis Breast Cancer	Family History		
		Any Cancers	Breast Cancer	NF1
1	29	+	+	+
2	39	+	+	+
3	39	+	unknown	unknown
4	41	+	+	-
5	43	+	-	-
6	44	+	+	+
7	49	+	+	unknown
8	49	+	+	-
9	57	+	-	+
10	70	+	unknown	unknown
11	unknown	+	+	+
12	unknown	+	+	-
13	34	-	-	-
14	37	-	-	unknown
15	40	-	-	+
16	43	-	-	+
17	47	-	-	+
18	47	-	-	-
19	49	-	unknown	+
20	unknown	-	unknown	unknown

+: Yes -: No

Table 4 Histological types of the breast cancer.

	Invasive (n)	in situ (n)	N/A (n)
Total	8	6	6
Ductal	6	6	8
ER+	4	0	13
ER--	2	1	
Her2+	1	1	15
Her2--	3	0	
Stage			
1	2		
2	1		
3	5		
4	0		

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Supplementary Table 1 Personal History of Breast Cancer or All Cancers and Clinical Features of NF1

			No Cancer		Cancer			No Cancer		Cancer		
		Feature Categories	N	%	N	%	P	N	%	N	%	P
Café-au-lait macules	All Cancers	0-5	33	10.34	6	5.71	0.32	33	11.07	6	6.25	0.237
		>=6	266	83.07	89	85.71		266	88.93	89	93.75	
		Missing info	21	6.58	9	8.57						
	Breast Cancer	0-5	38	9.45	1	4.55	0.111	38	10.11	1	5.56	1
		>=6	340	84.08	15	77.27		340	89.89	15	94.44	
		Missing info	26	6.47	3	18.18						
Lisch Nodule	All Cancers	0	105	32.92	34	32.38	0.462	105	51.47	34	46.58	0.498
		>=1	100	31.03	38	37.14		100	48.53	38	53.42	0
		Missing info	115	36.05	32	30.48						
	Breast Cancer	0	137	34.08	2	9.09	0.001	137	50.55	2	33.33	0.447
		>=1	136	33.33	2	18.18		136	49.45	2	66.67	0
		Missing info	131	32.59	15	72.73						
Dermal Neuro-fibroma	All Cancers	0-1	45	14.11	12	11.43	0.348	45	14.61	12	11.54	0.513
		>=2	264	82.45	91	87.62		264	85.39	91	88.46	
		Missing info	11	3.45	1	0.95						
	Breast Cancer	0-1	55	13.68	2	9.09	0.871	55	14.1	2	9.09	0.753
		>=2	337	83.33	18	90.91		337	85.9	18	90.91	
		Missing info	12	2.99	0	0						
Plexiform Neurofibroma	All Cancers	0	172	53.92	58	55.24	0.851	172	61.87	58	61.7	1
		>=1	107	33.23	35	34.29		107	38.13	35	38.3	
		Missing info	41	12.85	11	10.48						
	Breast Cancer	0	218	54.23	12	54.55	0.055	218	61.24	12	75	0.306
		>=1	140	34.33	2	18.18		140	38.76	2	25	
		Missing info	46	11.44	6	27.27						
Learning Disability	All Cancers	No	172	53.61	46	44.76	0.268	172	70.08	46	61.04	0.162
		Yes	73	22.88	30	28.57		73	29.92	30	38.96	
		Missing info	75	23.51	28	26.67						
	Breast Cancer	No	212	52.24	6	36.36	0.026	212	67.74	6	72.73	1
		Yes	100	24.88	3	13.64		100	32.26	3	27.27	
		Missing info	92	22.89	11	50						

Supplementary Table 1 Personal History of Breast Cancer or All Cancers and Clinical Features of NF1…continued

			No Cancer		Cancer			No Cancer		Cancer		
		Feature Categories	N	%	N	%	P	N	%	N	%	P
Vasculopathy	All Cancers	No	242	75.5	73	70.48	0.47	242	95.63	73	96.1	1
		Yes	11	3.45	3	2.86		11	4.37	3	3.9	0
		Missing info	68	21	27	26.67						
	Breast Cancer	No	307	75.87	8	45.45	0.002	307	95.61	8	100	1
		Yes	14	3.48	0	0		14	4.39	0	0	0
		Missing info	84	20.65	11	54.55						
Scoliosis	All Cancers	No	202	63.01	66	63.81	1	202	73.9	66	74.44	1
		Yes	72	22.26	22	21.9		72	26.1	22	25.56	0
		Missing info	47	14.73	15	14.29						
	Breast Cancer	No	259	64.18	9	45.45	0.023	259	74.14	9	71.43	0.763
		Yes	91	22.39	3	18.18		91	25.86	3	28.57	0
		Missing info	54	13.43	8	36.36						
Short Stature	All Cancers	No	129	40.75	43	41.9	0.538	129	76.47	43	72.13	0.494
		Yes	40	12.54	17	16.19		40	23.53	17	27.87	
		Missing info	151	46.71	42	41.9						
	Breast Cancer	No	164	40.8	10	45.45	0.539	164	74.55	10	90.91	0.301
		Yes	56	13.93	1	4.55		56	25.45	1	9.09	
		Missing info	184	45.27	9	50						
Macrocephaly or learning disability	All Cancers	No	147	46.08	45	42.86	0.849	147	55.47	45	52.33	0.62
		Yes	118	36.99	41	39.05		118	44.53	41	47.67	
		Missing info	55	16.93	18	18.1						
	Breast Cancer	No	184	45.77	8	36.36	0.003	184	54.28	8	66.67	0.558
		Yes	155	38.56	4	18.18		155	45.72	4	33.33	
		Missing info	64	15.67	9	45.45						
Bony Dysplasia	All Cancers	No	273	85.58	87	82.86	0.259	273	94.46	87	89.69	0.157
		Yes	16	5.02	10	9.52		16	5.54	10	10.31	
		Missing info	30	9.4	8	7.62						
	Breast Cancer	No	345	85.32	15	77.27	0.4	345	93.46	15	89.47	0.371
		Yes	24	5.97	2	9.09		24	6.54	2	10.53	
		Missing info	35	8.71	3	13.64						